AD_____

Award Number: DAMD17-02-1-0726

TITLE: Testing Whether Defective Chromatin Assembly in S-Phase Contributes to Breast Cancer

PRINCIPAL INVESTIGATOR: Peter D. Adams, Ph.D.

CONTRACTING ORGANIZATION: Fox Chase Cancer Center
Philadelphia, Pennsylvania 19111

REPORT DATE: October 2003

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

20040602 075

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	October 2003	Annual (30 Sep 2002 - 29 Sep 2003)

4. TITLE AND SUBTITLE
Testing Whether Defective Chromatin Assembly in S-Phase Contributes to Breast Cancer

5. FUNDING NUMBERS
DAMD17-02-1-0726

6. AUTHOR(S)
Peter D. Adams, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Fox Chase Cancer Center
Philadelphia, Pennsylvania 19111

E-Mail: pd_adams@fccc.edu

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES
Original contains color plates: All DTIC reproductions will be in black and white.

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT (Maximum 200 Words)
Cancer cells characteristically have a high frequency of genome rearrangements. Although these genome rearrangements are likely to contribute to the defective proliferation control that is characteristic of cancer cells, the cause of rearrangement is poorly understood. We used a dominant negative mutant of (chromatin assembly factor-I) CAF1, a complex that assembles newly synthesized DNA into nucleosomes, to inhibit S-phase chromatin assembly and found that this induced S-phase arrest. Arrest was accompanied by DNA damage. These results show, for the first time, that I human cells CAF1 activity is required for completion of S-phase and defects in chromatin assembly induce DNA damage. We propose that error in chromatin assembly, occurring spontaneously or cause d by genetic mutations or environmental agents, contribute to genome instability and cancer. Consistent with this idea, preliminary evidence indicates that chromatin assembly factors are mutated in some human cancers.

14. SUBJECT TERMS
Chromatin, genome stability, cancer

15. NUMBER OF PAGES
25

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

Table of Contents

Introduction

Cancer cells characteristically have a high frequency of genome rearrangements *(1)*. Although these genome rearrangements are likely to contribute to the defective proliferation control that is characteristic of cancer cells, the cause of rearrangements is poorly understood. We used a dominant negative mutant of (chromatin assembly factor-I) CAF1 *(2)*, a complex that assembles newly synthesized DNA into nucleosomes, to inhibit S-phase chromatin assembly and found that this induced S-phase arrest *(3)*. Arrest was accompanied by DNA damage. These results show, for the first time, that in human cells CAF1 activity is required for completion of S-phase and defects in chromatin assembly induce DNA damage. We propose that errors in chromatin assembly, occurring spontaneously or caused by genetic mutations or environmental agents, contribute to genome instability and cancer. Consistent with this idea, preliminary evidence indicates that chromatin assembly factors are mutated in some human cancers.

Body.

The original tasks are italicized below and addressed individually.

Task 1. To investigate the processes that monitor chromatin assembly in primary HMECs and determine if these monitoring processes are impaired in transformed breast cancer derived cell lines.

In the original application, we proposed that defects in chromatin assembly in S-phase activate a checkpoint that blocks on-going DNA synthesis - a so-called chromatin assembly checkpoint. We subsequently found, in the early stages of DOD funding, that defects in chromatin assembly cause DNA damage *(3, appendix)*. A large body of published data has shown that DNA damage causes S-phase arrest *(4)*. Consequently, we now favor the view that the S-phase arrest caused by defects in chromatin assembly is a consequence of DNA damage. This work has been published in *Molecular Cell (3)*. In light of this change in the model, most of our effort has been directed toward the work recently published in *Molecular Cell (3, appendix)* and Task 2.

a. Construct and test retroviruses expressing hair-pin siRNAs to inactivate CAF1, ASF1a and ASF1b (months 1-4).

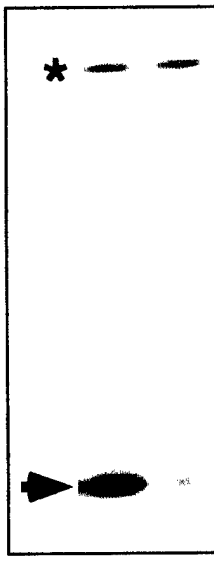


Figure 1. Primary human fibroblasts were infected with a control retrovirus or a retrovirus encoding an shRNA against ASF1a. Each virus also encoded resistance to puromycin. The cells were selected in puromycin for 3 days, extracts prepared and western blotted with antibodies to ASF1a. The arrowhead indicates the position of ASF1a and the asterix marks a non-specific protein that acts as a loading control.

We have successfully constructed a retrovirus that directs expression of an shRNA that knocks-down expression of ASF1a after infection of primary human fibroblasts (Figure 1). Having validated the technology, we are now constructing retroviruses that can be used to knock-down expression of ASF1b and CAF1. Using these retroviruses we will examine the effects of defects in chromatin assembly in primary breast epithelial cells and transformed breast cell lines (Task 1b and c). In particular, we will test whether defects in chromatin assembly contribute to transformation of primary breast epithelial cells (Task 2c).

b. Use siRNA expressing retroviruses (or alternative approaches described) to perturb chromatin assembly and determine the effect on cell cycle progression in primary

HMECs and transformed breast cancer cell lines (months 5-14).

No progress yet.

c. Use standard approaches to determine whether the effects of defective chromatin assembly in primary HMECs are mediated by a checkpoint (months 15-27).

No progress yet.

Task 2. To determine whether mutations in chromatin assembly factors occur in primary human breast cancer and whether defects in chromatin assembly contribute to transformation of primary HMECs in culture.

Before embarking on the major subtasks of Task 2, we chose to sequence cDNAs and genomic DNA derived from a panel of tumor-derived transformed human cell lines. The advantage of this approach are several: 1) the cDNAs and genomic DNA are readily available in essentially unlimited amounts; 2) the cell lines are uncontaminated by non-transformed cells; 3) we were able to start without waiting for IRB approval to use patient-derived material.

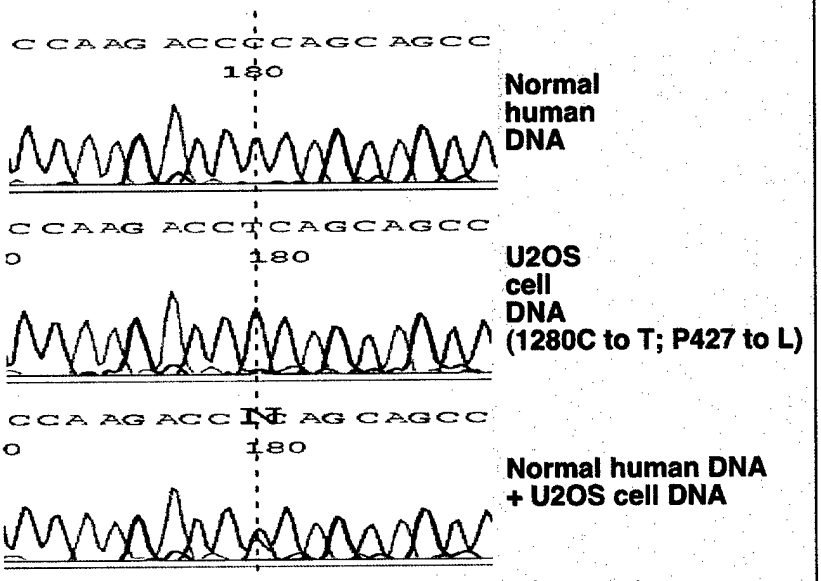


Figure 2. Genomic DNA from human U2OS cells (an osteosarcoma cell line) and normal human DNA was sequenced through the gene encoding p60CAF1A. In the bottom panel the two DNAs were mixed in equal amounts prior to sequencing. A somatic base change in the U2OS cell DNA is indicated by the dashed line.

We sequenced cDNAs encoding p60CAF1 from 17 human tumor-derived cell lines (including 2 breast cancer cell lines, MCF7 and MDAMB435). In one of these cell lines (U2OS cells - an osteosarcoma derived cell line), we found a C to T somatic base change that is predicted to result in substitution of P427 with L (P427L). This base change was confirmed by sequencing of the corresponding genomic DNA. Since U2OS cells are derived from a Caucasian individual, as a control we sequenced genomic DNA from 100 normal Caucasians with no cancer history. In all of these, residue 427 was predicted to be P. This argues against the P427L substitution being a polymorphic variant, although we cannot rule out a very rare polymorphism. Significantly, the DNA sequence data showed only a C at this position, and no wild type T. Thus, either U2OS cells are homozygous for the P427L variant, or the 2nd allele is deleted or otherwise not expressed. Since a homozygous, rare polymorphic variant seems highly unlikely, these data support the notion that the P427L variant has been selected for during tumor progression.

We are encouraged by these preliminary data and will now continue with the tasks described below.

a. Prepare cDNA from 20 primary breast tumors (months 1-2).

In progress. In addition, we are preparing cDNA from other tumor types, including osteosarcoma.

b. Sequence breast tumor cDNAs encoding ASF1a, ASF1b and p150CAF1 to determine whether they contain mutations. Where appropriate sequence cDNA derived from germline DNA to rule out polymorphisms (months 3-15).

No progress yet.

c. Infect primary HMECs with retroviruses designed to disrupt chromatin assembly and determine whether defective chromatin assembly contributes to transformation of primary HMECs (months 16-36).

No progress yet.

Key Research Accomplishments.

- 1. This is the first published demonstration that defects in chromatin assembly inhibit DNA synthesis and cause DNA damage in human cells. This finding predicts that defects in chromatin assembly will promote the genome instability that is thought to contribute to development of the neoplastic phenotype.
- 2. To our knowledge, this is the first preliminary evidence that S-phase chromatin assembly factors, such as CAF1, are mutated in human tumors.

Reportable Outcomes.

Manuscripts.

We have already reported key research accomplishment #1 in *Molecular Cell (3, appendix)*. In addition, this work has been extensively reviewed elsewhere *(5-7, appendix)*.

Meeting abstracts/presentations

Oral presentation at 2002 Cold Spring Harbor Cell Cycle meeting, Cold Spring Harbor, NY
Poster presentation at 2003 FASEB Chromatin meeting, CO

Invited seminars

2002 University of Pennsylvania, Philadelphia, PA
2003 Dana Farber Cancer Institute, Boston, MA

Conclusions.

As reported in *Molecular Cell (3, appendix)*, defects in chromatin assembly can contribute to DNA damage and genome instability in human cells. As predicted from this idea, preliminary evidence from sequence analysis of chromatin assembly factors in human tumor derived cell lines indicates that mutations in chromatin assembly factors might be selected for and drive progression of human tumors. Continuation of this project has the potential to uncover a novel class of tumor suppressor and oncogenes that might, ultimately, provide new diagnostic, prognostic and therapeutic targets.

References.

1. Lengauer, C., Kinzler, K.W., and Vogelstein, B., (1998) Genetic instabilities in human cancers. *Nature*, 396(6712), 643-649.
2. Tyler, J.K., (2002) Chromatin assembly. *Eur J Biochem*, 269(9), 2268-2274.
3. Ye, X., Franco, A.A., Santos, H., Nelson, D.M., Kaufman, P.D., and Adams, P.D., (2003) Defective S-phase chromatin assembly causes DNA damage, activation of the S-phase checkpoint and S-phase arrest. *Molecular Cell*, 11, 341-351.
4. Zhou, B.B. and Elledge, S.J., (2000) The DNA damage response: putting checkpoints in perspective. *Nature*, 408(6811), 433-439.
5. Ye, X. and Adams, P.D., (2003) Coordination of S-phase events and genome stability. *Cell Cycle*, 2(3), 185-187.
6. Verreault, A., (2003) Histone deposition at the replication fork: a matter of urgency. *Mol Cell*, 11(2), 283-284.
7. Heinrichs, A., (2003) A fork load. *Nature Reviews: Mol Cell Biol*, 4(4), 253.

Appendix.

Attached are references 3, 5, 6, and 7.

Molecular Cell, Vol. 11, 341–351, February, 2003, Copyright ©2003 by Cell Press

Defective S Phase Chromatin Assembly Causes DNA Damage, Activation of the S Phase Checkpoint, and S Phase Arrest

Xiaofen Ye,[1] Alexa A. Franco,[2,3] Hidelita Santos,[1] David M. Nelson,[1] Paul D. Kaufman,[2,3] and Peter D. Adams[1,*]
[1]Division of Basic Science
Fox Chase Cancer Center
Philadelphia, Pennsylvania 19111
[2]351 Donner Laboratory
Lawrence Berkeley National Laboratory
Berkeley, California 94720
[3]Department of Molecular and Cell Biology
229 Stanley Hall #3206
University of California, Berkeley
Berkeley, California 94720

Summary

The S phase checkpoint protects the genome from spontaneous damage during DNA replication, although the cause of damage has been unknown. We used a dominant-negative mutant of a subunit of CAF-I, a complex that assembles newly synthesized DNA into nucleosomes, to inhibit S phase chromatin assembly and found that this induced S phase arrest. Arrest was accompanied by DNA damage and S phase checkpoint activation and required ATR or ATM kinase activity. These results show that in human cells CAF-I activity is required for completion of S phase and that a defect in chromatin assembly can itself induce DNA damage. We propose that errors in chromatin assembly, occurring spontaneously or caused by genetic mutations or environmental agents, contribute to genome instability.

Introduction

Cancer cells characteristically have a high frequency of genome rearrangements (Lengauer et al., 1998), although the cause of rearrangements is poorly understood. Genome integrity during S phase of the cell cycle depends on the S phase checkpoint. This checkpoint is activated by DNA damage or stalled replication forks and inhibits ongoing DNA synthesis (Abraham, 2001; Osborn et al., 2002), thus giving time for DNA repair. DNA double-strand breaks caused by ionizing radiation (IR) activate ATM kinase, whereas stalled replication forks caused by hydroxyurea (HU) and lesions caused by ultraviolet (UV) light activate the related kinase, ATR. Downstream effectors of ATM and ATR include BRCA1, NBS1, Mre11, FANCD2, Chk1 and Chk2 kinases, the histone H2A variant, H2AX, and p53 (Abraham, 2001; Taniguchi et al., 2002; Redon et al., 2002). Underscoring the importance of the S phase checkpoint, many S phase checkpoint genes, such as ATM, NBS1, Mre11, BRCA1 (Khanna and Jackson, 2001), Chk2 (Bell et al., 1999), p53 (Vogelstein et al., 2000), and FANCD2 (Taniguchi et al., 2002), are mutated in human cancers.

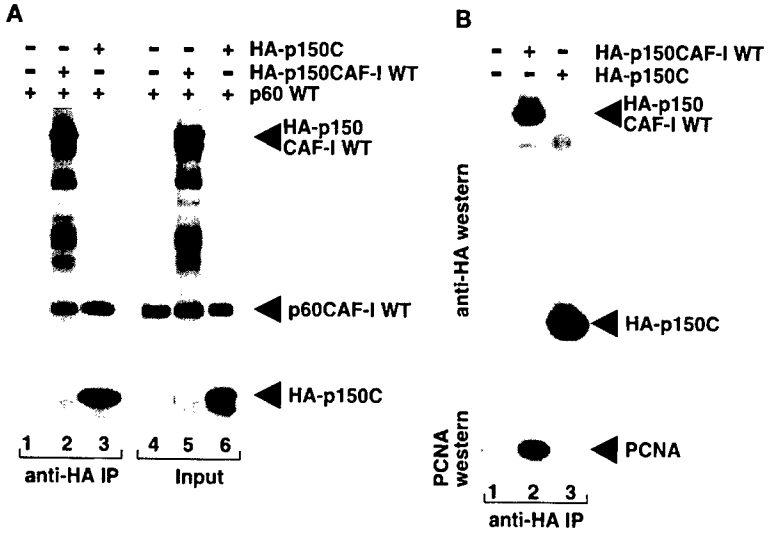
The S phase checkpoint also maintains genome stability in the absence of external genotoxic stress. Inactivation of ATR (Brown and Baltimore, 2000; de Klein et al., 2000), Chk1 (Liu et al., 2000; Takai et al., 2000), Hus1 (Weiss et al., 2000), BRCA1 (Hakem et al., 1996; Liu et al., 1996), NBS1 (Zhu et al., 2001), or Mre11 (Xiao and Weaver, 1997) in normal somatic cells is lethal, and mouse cells lacking ATR exhibit high levels of chromosome abnormalities (Brown and Baltimore, 2000; de Klein et al., 2000). In addition, it was recently shown that deficiency of ATR in mammalian cells causes expression of "fragile sites," characterized by formation of gaps and breaks on metaphase chromosomes (Casper et al., 2002). Depletion of xMre11 from *X. laevis* cell-free extracts causes the accumulation of double-strand breaks in S phase (Costanzo et al., 2001). In yeast, mutant alleles, such as *mec1*, *mre11*, *chk1*, and *rad53* (inactivated yeast homologs of human ATR/ATM, Mre11, Chk1, and Chk2, respectively), cause spontaneous "gross chromosomal rearrangements" (GCRs) (Kolodner et al., 2002). In sum, the S phase checkpoint protects against spontaneous DNA damage that arises in a normal S phase.

One likely source of spontaneous damage is stalled replication forks that are processed to Holliday junctions and double-strand breaks (Osborn et al., 2002). A cell's response to stalled forks depends on the S phase checkpoint. In yeast, the checkpoint is required to reinitiate DNA replication after transient HU-mediated arrest (Desany et al., 1998), to maintain stable replication forks in the presence of an HU-mediated arrest (Lopes et al., 2001), and to prevent collapse of replication forks in response to methyl methanesulphonate (MMS)-mediated DNA damage (Tercero and Diffley, 2001). In the absence of exogenous DNA-damaging agents, Mec1 promotes fork progression through "replication slow zones," where forks have a tendency to stall (Cha and Kleckner, 2002)

Electron microscopy studies showed that in wild-type yeast, stalled forks retain a bifurcated, Y-shaped appearance. In cells lacking the S phase checkpoint, however, stalled forks frequently reversed to form Holliday junction-like "chickenfoot" structures that, by inappropriate processing, could give rise to double-strand breaks (Sogo et al., 2002). Therefore, the S phase checkpoint acts to prevent stalling and collapse of replication forks and, consequently, DNA damage and genome instability. The factors that influence the frequency of stalled forks are poorly understood.

In S phase, nucleosomes are assembled onto newly synthesized DNA within a few hundred base pairs of the fork by chromatin assembly factors, including CAF-I and ASF1 (Tyler, 2002). CAF-I is a heterotrimeric complex consisting of p150CAF-I, p60CAF-I, and p48CAF-I (Smith and Stillman, 1989). Direct binding of p150CAF-I to the replication processivity protein, PCNA, targets CAF-I to sites of DNA synthesis and contributes to coupling of DNA synthesis and chromatin assembly (Krawitz et al., 2002; Marheineke and Krude, 1998; Martini et al., 1998; Moggs et al., 2000; Shibahara and Stillman, 1999). We showed recently that repression of histone synthesis triggers S phase arrest in human cells, suggesting that

*Correspondence: pd_adams@fccc.edu



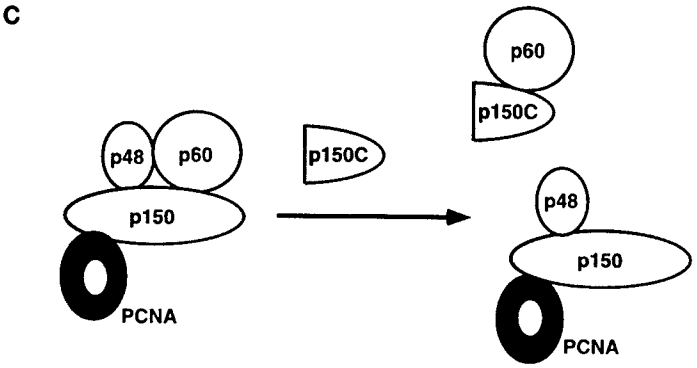


Figure 1. HA-p150C Is a Putative Dominant-Negative Inhibitor of CAF-I

(A) ^{35}S-labeled p60CAF-I was in vitro translated with or without cotranslation of HA-p150CAF-IWT or HA-p150C as indicated. Reactions were immunoprecipitated with an anti-HA antibody (12CA5, lanes 1–3) and fractionated by SDS-PAGE. Lanes 4–6 contain 20% of input proteins.

(B) U2OS cells were transiently transfected with pcDNA3 HA-p150CAF-I WT or HA-p150C as indicated. Cell lysates were immunoprecipitated with an anti-HA antibody (Y11) and Western blotted with antibodies to HA (12CA5) or PCNA (PC10) as indicated.

(C) A model for the proposed mode of action of HA-p150C (see text for details).

DNA synthesis and chromatin assembly are obligatorily coupled (Nelson et al., 2002). Here, we directly tested whether disruption of S phase chromatin assembly affected DNA synthesis by inhibition of CAF-I. Indeed, inhibition of CAF-I blocked DNA synthesis, induced DNA damage, and activated the S phase checkpoint. These results suggest that errors in chromatin assembly, either spontaneous or resulting from genetic mutations or environmental agents, are likely to increase the rate of DNA mutation and genome instability.

Results

A Dominant-Negative Mutant of p150CAF-I

As described previously, both HA-p150CAF-IWT and HA-p150C bound stably to p60CAF-I (Figure 1A), whereas only HA-p150CAF-IWT bound stably to PCNA (Figure 1B) (Kaufman et al., 1995; Moggs et al., 2000). We reasoned that HA-p150C could behave as a dominant-negative inhibitor of chromatin assembly by CAF-I via titration of p60CAF-I into nonfunctional complexes (Figure 1C).

During replication-coupled, CAF-I-dependent chromatin assembly assays in vitro, incorporation of newly replicated plasmid DNA into nucleosomes causes the DNA to become negatively supercoiled (Smith and Stillman, 1989). In this assay, HA-p150C inhibited CAF-I-dependent chromatin assembly (Figures 2A and 2B). There was no effect on DNA synthesis (production of ^{32}P-labeled plasmid), indicating that it does not perturb the progression of replication forks directly. Importantly, inhibition of nucleosome formation by HA-p150C was abolished by excess purified, recombinant human CAF-I (Figure 2B), confirming that HA-p150C acts as a specific inhibitor of CAF-I.

We predicted that overexpression of HA-p150C in human cells would disrupt the interaction between endogenous p60CAF-I and p150CAF-I (Figure 1C). Indeed, endogenous p150CAF-I coprecipitated with endogenous p60CAF-I in the absence but not the presence of ectopically expressed HA-p150C (Figure 2C). Additionally, expression of HA-p150C in cells resulted in a dramatic reduction in the total amount of p150CAF-I (Figure 2C, lanes 5–8), suggesting that p150CAF-I is degraded when not incorporated into the CAF-I complex. Expression of p60CAF-I was unaffected by HA-p150C (Figure 2D). However, p60CAF-I was stably bound to chromatin in punctate DNA replication foci in 45% of control cells but only 15% of the cells expressing HA-p150C (Figure 2E; see note in Experimental Procedures). These data indicate that HA-p150C disrupts the interaction between endogenous p150CAF-I and p60CAF-I and prevents tight association of p60CAF-I with chromatin and sites of DNA synthesis.

Inhibition of DNA Synthesis

We next tested whether HA-p150C affected DNA synthesis in vivo. As shown in Figure 3A, mock-transfected cells released synchronously into S phase progressed normally through S phase. In contrast, cells transiently

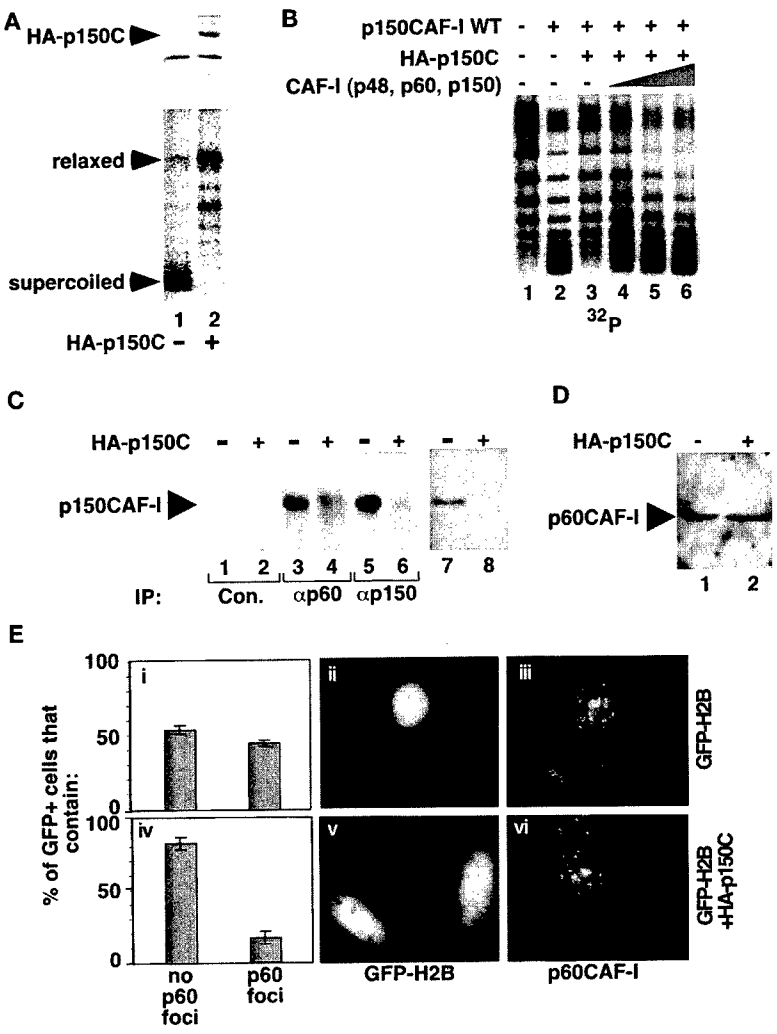


Figure 2. HA-p150C Inhibits CAF-I-Dependent Chromatin Assembly In Vitro and Disrupts the Endogenous CAF-I Complex In Vivo

(A) DNA replication-coupled CAF-I-dependent chromatin assembly assays were performed with purified p150CAF-I and an in vitro translation reaction that was unprogrammed (lane 1) or expressed HA-p150C (lane 2). HA-p150C was detected by Western blotting with anti-HA (12CA5, upper panel). Purified ^{32}P-labeled replicated plasmid DNA was fractionated by agarose gel electrophoresis (lower panel) to resolve relaxed (nonnucleosomal) and supercoiled (nucleosomal) plasmids.

(B) DNA replication-coupled CAF-I-dependent chromatin assembly assays were performed in the absence or presence of ~4 ng p150CAF-IWT, in vitro-translated HA-p150C, and purified recombinant trimeric CAF-I complex (5.5, 11, or 22 ng as indicated by the triangle).

(C) U2OS cells were transiently transfected with pCMV-CD19 together with pcDNA3 HA-p150C or pcDNA3 as indicated. Transfected cells were immunopurified with anti-CD19 coated magnetic beads, and lysates were prepared and immunoprecipitated with antibodies to p60CAF-I (SS24), p150CAF-I (SS1), or control (419) as indicated, fractionated by SDS-PAGE, and Western blotted with an antibody to p150CAF-I (SS1). Lanes 7 and 8 contain 150 μg of whole-cell lysate derived from immunopurified transfected cells.

(D) Lysates from (C) were Western blotted with antibodies to p60CAF-I (a cocktail of SS3, SS53, SS60, and SS96).

(E) U2OS cells were transiently transfected with pBOS-GFP-H2B in the absence (Ei–Eiii) or presence (Eiv–Evi) of pCDNA3 HA-p150C. The cells were stained with antibodies to p60CAF-I (SS75) and visualized by immunofluorescence microscopy to detect GFP-H2B and p60CAF-I. 100 cells were counted and scored as p60CAF-I positive or negative. The results of two independent experiments are plotted in (Ei) and (Eiv).

transfected with a plasmid encoding HA-p150C had a profound defect in S phase progression. Many failed to detectably exit G1 phase, and most of those that did arrested within S phase. We also measured DNA synthesis by pulse labeling with 5′-BrdU at a time when the cells had accumulated in S phase. Most of the HA-p150C-expressing cells failed to incorporate 5′-BrdU and thus were not actively synthesizing DNA (Figure 3B). (Thirteen percent of HA-p150C-expressing cells were 5′-BrdU positive compared to 56% of the untransfected cells on the same coverslip; these results are representative of more than five similar experiments.) Therefore, both FACS analysis and 5′-BrdU labeling demonstrated that HA-p150C inhibited DNA synthesis. In contrast, full-length HA-p150CAF-IWT failed to inhibit DNA synthesis (Figures 3D and 3E), and coexpression of HA-p150CAF-IWT with HA-p150C abolished the arrest (Figures 3F and 3G). These data confirm that the effect of HA-p150C on DNA synthesis depends on its ability to perturb the endogenous CAF-I complex. Significantly, the cell cycle arrest induced by HA-p150C was indistinguishable from the arrest induced by ectopic expression of human HA-HIRA (Figure 3C), a protein whose ectopic expression represses histone gene expression, thus indirectly inhibiting chromatin assembly (Hall et al., 2001; Nelson et al., 2002).

The model in Figure 1C predicts that overproduction of any fragment of p150CAF-I that binds p60CAF-I but not PCNA will inhibit DNA synthesis. Consistent with this idea, HA-p150CAF-I(451-938), which did not bind PCNA, efficiently induced arrest, but HA-p150CAF-I(250-938), which did bind to PCNA, failed to induce arrest (Figures 4A and 4C). These data also indicated that the PCNA binding domain of HA-p150CAF-IWT is between residues 250 and 451, consistent with previous sequence analysis which identified a partial consensus PCNA binding domain at residues 421–431 (Krawitz et al., 2002). As anticipated, deletion of residues 421–431 produced a polypeptide (HA-p150CAF-IΔPCNA) that bound to p60CAF-I but not PCNA and inhibited DNA synthesis (Figures 4A–4C). Expression of HA-p150CAF-IΔPCNA did not affect localization of PCNA to DNA replication foci (Figure 4D; see note in Experimental Procedures) (Bravo and Macdonald-Bravo, 1985; Celis and Celis, 1985). Thus, perturbation of CAF-I inhibits DNA synthesis but, as far as we can tell from Figure 4D, does not affect assembly of DNA replication foci.

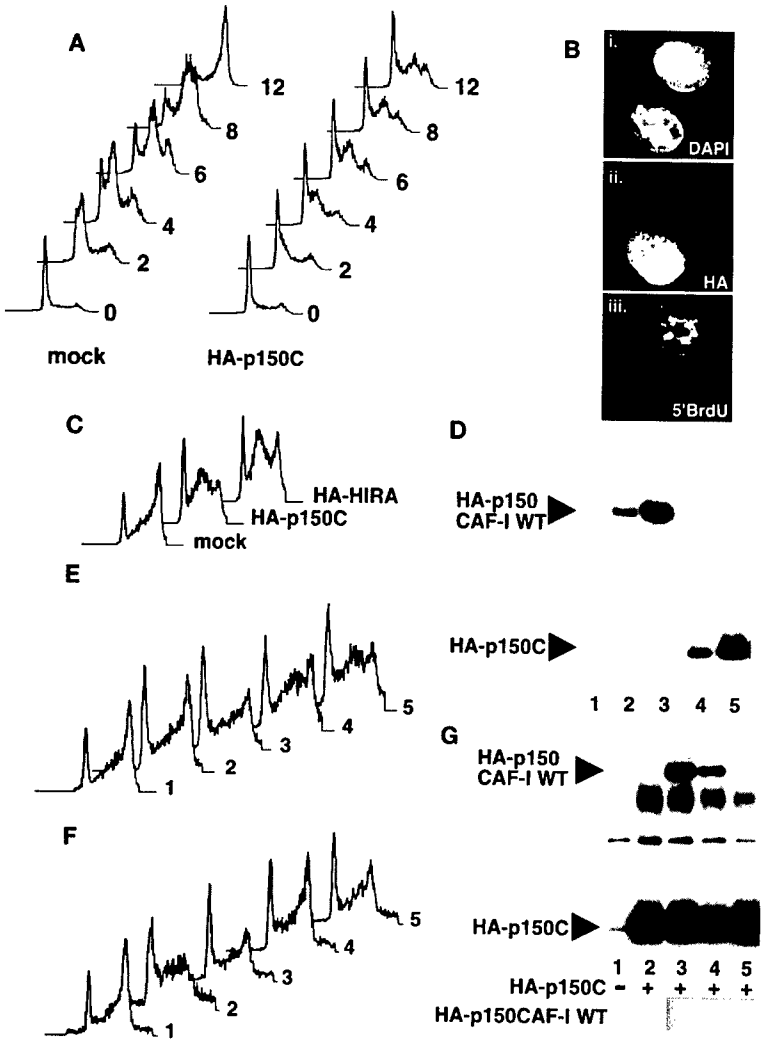


Figure 3. HA-p150C Blocks DNA Synthesis and Progression through S Phase

(A) U2OS cells were transiently transfected with pCMV CD19 together with pcDNA3 or pcDNA3 HA-p150C as indicated. 16 hr later, the cells were arrested in mimosine for 20 hr and released into S phase, and at time intervals afterwards, the cell cycle distribution of the CD19$^+$ cells was determined by FACS.

(B) U2OS cells were transiently transfected with pcDNA3 HA-p150C, and 36 hr later were pulse labeled for 30 min with 10 μM 5′-BrdU and stained with DAPI to visualize the DNA (Bi), anti-HA (Y11) (Bii), and anti-5′-BrdU-FITC (Biii).

(C) U2OS cells were transiently transfected with pCMV CD19 together with pcDNA3, pcDNA3 HA-p150C, or pcDNA3 HA-HIRA, as indicated, and processed as in (A).

(D) U2OS cells were transfected with pCMV-CD19 together with pcDNA3 (lane 1), 14 and 28 μg of pcDNA3 HA-150CAF-IWT (lanes 2 and 3, respectively), or 1 and 3 μg of pcDNA3 HA-p150C (lanes 4 and 5, respectively). 5 μg of whole-cell lysate was fractionated by SDS-PAGE and Western blotted with anti-HA (12CA5). Lanes 1–3 and 4–5 of are nonadjacent lanes from the same exposure of the same gel.

(E) The same transfections as (D) processed as in (A).

(F) U2OS cells were transfected with pCMV CD19 together with pcDNA3 HA-p150C or pDNA3, as indicated, and decreasing amounts of pcDNA3 HA-p150CAF-IWT (as indicated by the shaded triangle in [G]). The cells were processed as in (A).

(G) 5 μg of whole-cell lysate from cells in (F) was fractionated by SDS-PAGE and Western blotted with anti-HA (12CA5).

Additionally, we predicted that fragments of HA-p150CAF-I that fail to bind to both PCNA and p60CAF-I should fail to induce S phase arrest. As anticipated, deletion of the C-terminal p60CAF-I binding site (Kaufman et al., 1995) from HA-p150CAF-IΔPCNA protein resulted in a polypeptide, HA-p150CAF-IΔPCNA(1-547), that failed to bind to either p60CAF-I or PCNA and did not induce S phase arrest (Figures 5A and 5B).

We expected that the cells arrested in S phase would have defective chromatin structure. Digestion with micrococcal nuclease (MNase) was used to probe the chromatin structure of cells in S phase (Nelson et al., 2002). Chromatin from HA-p150CAF-I(451-938)-expressing cells was indeed more sensitive to digestion than chromatin from mock-transfected control cells (Figures 5C and 5D). We conclude that disruption of the endogenous CAF-I complex induces chromatin abnormalities.

Induction of DNA Damage

One cause of S phase arrest is DNA damage (Abraham, 2001). An early response to DNA damage, particularly double-strand breaks, is phosphorylation of histone H2AX in chromatin surrounding the lesion (Redon et al., 2002). To determine whether the DNA in arrested cells contained double-strand breaks, we tested whether H2AX was phosphorylated. Phosphorylated H2AX (γH2AX) staining was enriched in HA-p150C- and HA-p150CAF-IΔPCNA-expressing cells relative to the untransfected cells or cells expressing HA-p150CAF-IWT (Figures 6A and 6B). Significantly, nuclei that had the brightest γH2AX staining often contained visibly abnormal DAPI-stained nuclear structures, consistent with a defect in chromatin assembly and/or extensive DNA fragmentation (Figure 6A).

In addition, even without MNase treatment, the DNA extracted from HA-p150CAF-I(451-938)-expressing cells reproducibly migrated faster on agarose gels than DNA from mock-transfected cells or cells transfected with HA-p150CAF-I (Figures 5C and 6C; data not shown). However, at the same time after transfection (36 hr), the dominant-negative fragments of HA-p150CAF-I did not detectably induce three characteristic markers of apoptosis: chromatin condensation, cleavage of genomic DNA into a "nucleosomal ladder," and cells with less than 2n DNA content (Loo and Rillema, 1998) (Figures 6 and 3C; data not shown). These data suggest that

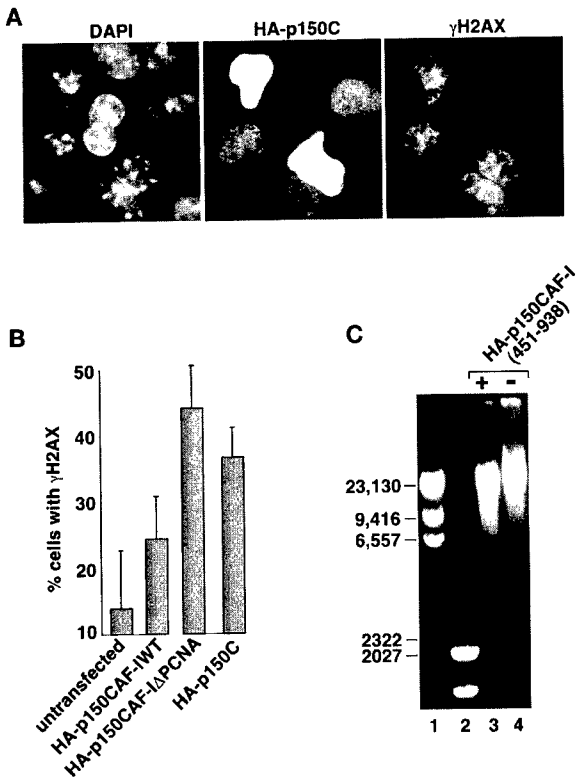


Figure 6. Dominant-Negative HA-p150CAF-I Mutants Induce DNA Damage

(A) U2OS cells on coverslips were transiently transfected with pcDNA3 HAp150C. The cells were stained with DAPI, anti-γH2AX antibodies, or anti-HA antibodies, as indicated, and visualized by immunofluorescence microscopy.

(B) 100 transfected HA^+ cells or 100 untransfected HA^- cells from the same coverslip were scored as positive or negative for γH2AX. The results are the mean of 12 (untransfected), 8 (HA-p150CAF-IWT and HA-p150C), or 2 (HA-p150CAF-IΔPCNA) independent experiments.

(C) U2OS cells were transiently transfected with pcDNA3 (lane 4) or pcDNA3 HAp150CAF-I(451-938) (lane 3) together with pCMV CD19. The cells were processed, and genomic DNA was purified as in lanes 2 and 3 of Figure 5C (without MNase). Lane 1 contains 1 μg of phage λ DNA digested with HindIII (MW markers indicated on left of gel), and lane 2 contains 1 μg of a 100 bp ladder.

cells. We propose that inhibition of S phase chromatin assembly causes stalled replication forks, which are inappropriately processed to DNA double-strand breaks. Most likely, stalled forks and double-strand breaks are responsible for ATR- and ATM-dependent checkpoint activation and cell cycle arrest.

CAF-I Is Required for S Phase Progression

Several lines of evidence indicate that inhibition of DNA synthesis by HA-p150C and HA-p150CAF-IΔPCNA results from inhibition of CAF-I-dependent chromatin assembly. First, HA-p150C inhibits chromatin assembly but not DNA synthesis in vitro, disrupts the interaction between endogenous p150CAF-I and p60CAF-I in vivo, and blocks stable association of p60CAF-I with replication foci in vivo (Figure 2). Second, HA-p150C-induced inhibition of DNA synthesis is abolished by coexpression of HA-p150CAF-IWT, just as excess trimeric CAF-I complex restores nucleosome assembly to HA-p150C-inhibited reactions in vitro (Figures 2 and 3). Third, the ability of HA-p150CAF-IWT and HA-p150CAF-I fragments to inhibit cell cycle progression correlates inversely with their ability to bind PCNA (Figure 4). Fourth, inhibition of DNA synthesis is not due to total disruption of DNA replication foci, because in arrested cells, PCNA was still localized in a punctate pattern characteristic of normal replication foci (Figure 4D). Fifth, the ability of HA-p150CAF-I fragments to inhibit cell cycle progression appears to depend upon their ability to sequester endogenous p60CAF-I away from the endogenous p150CAF-I/PCNA complex, because a mutant that fails to bind to both PCNA and p60CAF-I is inert (Figure 5). Finally, chromatin from S phase cells expressing a dominant-negative HA-p150CAF-I fragment was more sensitive to MNase digestion than chromatin from control S phase cells, showing directly that inhibition of CAF-I in S phase induces defects in chromatin structure (Figure 5C). Together, these data strongly suggest that disruption of CAF-I-dependent chromatin assembly is responsible for inhibition of DNA synthesis.

Cell cycle arrest induced by HA-p150C and HA-p150CAF-IΔPCNA is similar to that induced by human HIRA (Hall et al., 2001; Nelson et al., 2002) (Figure 3C). Previously, we showed that repression of histone synthesis was the direct cause of HIRA-induced S phase arrest (Nelson et al., 2002). Therefore, direct inhibition of chromatin assembly by HA-p150C and HA-p150CAF-IΔPCNA or, presumably, indirect inhibition of chromatin assembly by HIRA-mediated repression of histone synthesis both block S phase DNA synthesis in human cells. Importantly, the fact that HA-p150C and repression of histone synthesis have identical effects on S phase very strongly suggests that chromatin assembly, rather than DNA synthesis per se, is the target of HA-p150C.

Previous investigations suggested that inhibition of chromatin assembly would not block DNA synthesis in S phase. First, in yeast none of the likely DNA synthesis-linked chromatin assembly factors identified to date, such as CAF-I, Asf1, or the Hir proteins, is essential for viability either alone or in combination (Kaufman et al., 1998; Sharp et al., 2001; Tyler et al., 1999). Second, in vitro replication of plasmid DNA in mammalian cell extracts or *D. melanogaster* embryo extracts does not require chromatin assembly (Bulger et al., 1995; Stillman, 1986). Third, yeast expressing histones from conditional promoters replicate their entire genome when new histone synthesis and chromatin assembly are blocked (Han et al., 1987; Kim et al., 1988). Fourth, in *C. elegans* and *D. melanogaster* embryos, the reduced histone synthesis caused by mutant alleles of SLBP have no obvious effect on DNA synthesis (Sullivan et al., 2001; Kodama et al., 2002). Fifth, although in *X. laevis* perturbation of CAF-I blocks development past the mid-blastula transition, it has no detectable effect on a somatic cell line (Quivy et al., 2001). In contrast, our data demonstrate that in intact human somatic cells, inhibition of chromatin assembly blocks DNA synthesis in S phase. Thus, DNA synthesis and chromatin assembly appear to be more tightly coupled in intact human cells than in other model organisms.

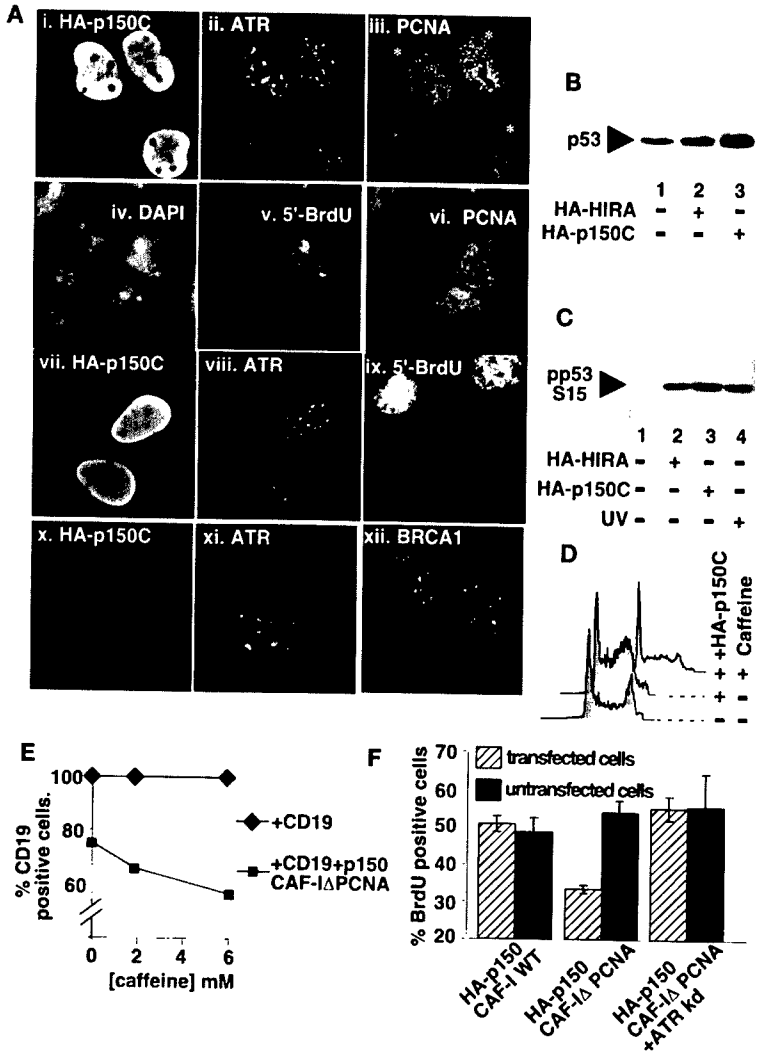


Figure 7. S Phase Arrest Depends upon Activation of the S Phase Checkpoint

(A) U2OS cells on coverslips were transiently transfected with pcDNA3 HA-p150C. In (Aiv)–(Aix), cells were pulse labeled for 15 min with 5′-BrdU prior to harvesting. Cells were stained with antibodies to HA, ATR, and PCNA (Ai–Aiii); 5′-BrdU and PCNA (Aiv–Avi); HA, ATR, and 5′-BrdU (Avii–Aix); and HA, ATR, and BRCA1 (Ax–Axii).

(B) U2OS cells were transiently transfected with pcDNA3 (lane 1), pcDNA3 HA-HIRA(421-729) (lane 2), or pcDNA3 HAp150C (lane 3) together with pCMV CD19. CD19$^+$ cells were immunopurified with magnetic beads, and cell lysates were Western blotted with an antibody to p53.

(C) Cell lysates from (B) were Western blotted with an antibody to p53pSer15. Lane 4 contains lysate from cells irradiated with UV light.

(D) U2OS cells were transiently transfected with a plasmid, pCMV CD19, in the absence or presence of pcDNA3 HA-p150C. 16 hr later, the cells were treated with 1.7 mM caffeine or PBS, and 24 hr later, the cell cycle distribution of the CD19$^+$ cells was determined.

(E) U2OS cells were transiently transfected with pCMV CD19 in the absence or presence of pcDNA3 HA-p150CAF-IΔPCNA. 36 hr later, the cells were treated with 2 or 6 mM caffeine or PBS, and 24 hr later, the percentage of the CD19$^+$ cells was determined by FACS. The number of CD19$^+$ HA-p150CAF-IΔPCNA-expressing cells is expressed as a percentage of the control (CD19 alone) at each dose of caffeine.

(F) ATM$^{-/-}$ human fibroblasts were transiently transfected with pcDNA3 HA-p150CAF-I WT or HA-p150CAF-I ΔPCNA in the absence or presence of a pcDNA3 ATRkd as indicated. 36 hr later, the cells were pulse-labeled for 1 hr with 10 μM 5′-BrdU and stained with anti-HA(Y11) and anti-5′-BrdU and DAPI. 100 untransfected cells (HA-negative) and 100 transfected cells (HA-positive) were scored as 5′-BrdU positive or negative and expressed as the percentage of 5′-BrdU positive. Each bar with standard deviation is the mean of four separate experiments.

Defects in Chromatin Assembly Cause DNA Damage

Inhibition of chromatin assembly induces DNA double-strand breaks as measured by accumulation of γH2AX and fragmentation of genomic DNA (Figure 6). These breaks are likely to result from inappropriate processing of stalled replication forks (Osborn et al., 2002). Several scenarios that result in damage are possible. First, PCNA binds to both replication and chromatin assembly proteins, such as DNA polymerase δ and p150CAF-I, respectively (Warbrick, 2000), suggesting that replication and chromatin assembly machineries function as an integrated complex in which disruption of one affects the other. Second, failure to package newly replicated DNA into chromatin might result in steric constraints that impede fork progression. Third, inhibition of chromatin assembly might not increase the rate of fork stalling but the frequency with which stalled forks are processed to double-strand breaks. Failure to incorporate the newly synthesized DNA into nucleosomes might increase the frequency of fork reversal, formation of a "chickenfoot," and resolution of this structure to give a double-strand break (Osborn et al., 2002; Sogo et al., 2002).

Checkpoint Activation

Several lines of evidence indicate that inhibition of chromatin assembly activates the S phase checkpoint. First, H2AX, a known substrate of ATR and ATM kinases, is phosphorylated (Figures 6A and 6B) (Redon et al., 2002). Second, ATR is recruited into nuclear foci, an indicator of ATR activation (Figure 7A) (Tibbetts et al., 2000). Third, p53 is stabilized and phosphorylated on serine 15, a known ATR and ATM phosphorylation site (Figures 7B and 7C) (Banin et al., 1998; Canman et al., 1998; Tibbetts et al., 1999). Fourth, the S phase BRCA1 foci in HA-p150C-expressing cells that contain ATR foci are dispersed, a marker of BRCA1 activation (Figure 7A) (Scully et al., 1997). Fifth, inactivation of the S phase checkpoint abolishes the S phase arrest and decreases cell viability (Figure 7D–7F), suggesting that a failure to arrest DNA synthesis is lethal to the cell. Taken together, these results show that inhibition of chromatin assembly activates the S phase checkpoint.

Conceivably, the defect in chromatin assembly might be directly responsible for checkpoint activation. Perhaps consistent with this idea, it was recently shown

that treatments that perturb chromatin structure without apparently inducing DNA damage, such as hypotonic buffer, chloroquine or trichostatin A, activate ATM kinase (Bakkenist and Kastan, 2003). However, the simplest model to explain checkpoint activation is that defects in chromatin assembly cause stalling of replication forks and double-strand breaks (Figure 6) and that these structures activate the ATR- and ATM-dependent checkpoints, similar to HU and IR (Abraham, 2001).

Chromatin Assembly and Genome Stability

Cancer cells are characterized by a high frequency of genome abnormalities (Lengauer et al., 1998), including "gross chromosomal rearrangements" (GCRs), such as translocations and large deletions. In the absence of an S phase checkpoint, progression though a normal S phase is associated with a high frequency of GCRs (Kolodner et al., 2002). This suggests that S phase is an inherently mutagenic process and that one function of the checkpoint is to suppress formation of GCRs. In addition, the S phase checkpoint prevents stalling of replication forks and stabilizes them after they have stalled (Cha and Kleckner, 2002; Lopes et al., 2001; Sogo et al., 2002; Tercero and Diffley, 2001). Since stalled replication forks can be processed to double-strand breaks, which are a potent source of GCRs (Osborn et al., 2002), it seems likely that the S phase checkpoint suppresses GCRs, at least in part, by protecting the integrity of replication forks and preventing conversion of stalled forks to double-strand breaks (Kolodner et al., 2002). However, the processes that influence replication fork stalling and formation of double-strand breaks in a normal S phase are largely unknown.

We propose that defects in S phase chromatin assembly cause double-strand breaks due to stalling and inappropriate processing of replication forks and that the S phase checkpoint limits the damage caused by defective chromatin assembly by stabilizing the stalled replication forks, inhibiting further DNA synthesis, and promoting DNA repair. If so, defects in chromatin assembly and inactivation of the S phase checkpoint should act synergistically to increase DNA damage. Indeed, Kolodner and coworkers have observed a synergistic effect in yeast on accumulation of GCRs due to mutations in the S phase checkpoint and chromatin assembly factors, such as *cac1* and *asf1* (K. Myung et al., personal communication).

In human cells, errors in chromatin assembly combined with inactivation of the S phase checkpoint might promote genome instability and neoplastic transformation. Several components of the S phase checkpoint, such as ATM, BRCA1, NBS1, Mre11 (Khanna and Jackson, 2001), Chk2 (Bell et al., 1999), p53 (Vogelstein et al., 2000), and FANCD2 (Taniguchi et al., 2002), are known to be mutated in human cancer. Errors in chromatin assembly might occur spontaneously or result from genetic mutations or environmental agents that inhibit chromatin assembly factors. Admittedly, the phenotype reported here may represent an extreme case that results from near-total inactivation of CAF-I, and such a profound phenotype is, presumably, lethal in most cell contexts. However, more subtle defects in chromatin assembly that result from haploinsufficiency of chromatin assembly factors or from point mutations within the PCNA binding site of p150CAF-I (that weaken, but do not completely disrupt, the p150CAF-I/PCNA interaction) might be expected to be nonlethal but increase the error rate associated with DNA replication. Consistent with this idea, some genes encoding chromatin assembly factors, such as p150CAF1, p48CAF1, ASF1a, and ASF1b, are located in regions of chromosomes reported to be deleted in some cancers (19p13, 1p34, 6q22, and 19p13, respectively; Couch and Weber, 2000; Mertens et al., 1997; Oesterreich et al., 2001; Sheng et al., 1996). We are currently testing whether these genes are the targets of mutations in human cancers.

Experimental Procedures

Cell Culture and Transfections

U2OS cells were cultured, transfected, and synchronized as described previously (Adams et al., 1996; Nelson et al., 2002).

Plasmids

pcDNA3 Flag-ATRkd was a gift of Drs. Robert Abraham and Kathy Brumbaugh. pBOS-GFP-H2B was purchased from Becton-Dickinson. All other plasmids were generated using standard molecular biology procedures, and details are available on request.

CAF-I-Dependent In Vitro Chromatin Assembly Assays

In vitro SV40 DNA replication/nucleosome assembly assays were performed as described (Kaufman et al., 1995). The three-subunit CAF-I complex and the p150CAF-I subunit were produced in insect cells and purified as described (Kaufman et al. 1995). HA-p150C was expressed by in vitro translation using TnT T7 Quick for PCR DNA (Promega).

Immunological Techniques

Anti-HA (12CA5, mouse monoclonal) was purchased from Roche. Anti-HA (Y11, rabbit polyclonal), anti-PCNA (PC10 and FL261), and anti-ATR (C19) were purchased from Santa Cruz Biotech. Anti-p53 (Ab6) and anti-BRCA1 (Ab1) were purchased from Oncogene Research Products, and anti-p53pS15 was purchased from Cell Signaling. Anti-5′-BrdU-FITC was purchased from Becton-Dickinson. Anti-γH2AX was purchased from UBI. Anti-CD19-FITC was purchased from Caltag. Anti-p150CAF-I and p60CAF-I have been described previously (Smith and Stillman, 1991). Immunoprecipitation and Western blots were performed as described previously (Adams et al., 1996). When performing anti-HA immunoprecipitations followed by anti-HA Western blots, mouse (12CA5) and rabbit (Y11) anti-HA antibodies were used so as to avoid detection of antibody heavy chain in the Western blot.

Immunofluorescence was performed as described previously by us or others (Hall et al., 2001; Tibbetts et al., 2000). Detailed methods of two- and three-color immunofluorescence are available upon request. To optimize the detection of p60CAF-I and PCNA by immunofluorescence, it was necessary to preextract the cells with EBC (Adams et al., 1996). Under these conditions, HA-p150C and p60CAF-I not stably bound to chromatin were washed out of the cells. GFP-H2B served as an NP40-resistant marker of transfected cells (Figures 2E and 4D). GFP-H2B did not affect cell cycle- nor HA-p150C-induced S phase arrest (data not shown) (Kanda et al., 1998).

Collection and FACS of CD19$^+$-Transfected Cells

Collection using magnetic beads and FACS was described previously (Adams et al., 1996; Nelson et al., 2002).

Digestion with MNase and Purification of Genomic DNA

Transiently transfected (CD19$^+$) cells were collected with anti-CD19 coated beads. Permeabilized nuclei were prepared and treated with Mnase, and genomic DNA was purified as described previously (Hall et al., 2001; Nelson et al., 2002).

Acknowledgments

We thank Dr. Richard Kolodner for communication of unpublished results and anonymous reviewers for insightful comments. We thank Dr. Bruce Stillman for p150CAF-I and p60CAF-I antibodies, Dr. Tim Yen for ATM$^{-/-}$ fibroblasts, Drs. Robert Abraham and Kathy Brumbaugh for ATRkd, Dr. Bin-bing Zhou for advice, and Drs. Randy Strich and Song-Tao Liu for critical reading of the manuscript. The work of P.D.A. is supported by NIH RO1-GM62281. A.A.F. was supported by an NSF predoctoral training grant, an NIH predoctoral training grant, and NIH grant 1R01 GM55712 to P.D.K. P.D.K. was supported by the California Breast Cancer Research Program grant number 4JB-0089, Department of Energy Field Work Proposal KP110301, and by Department of Energy funds administered through the Lawrence Berkeley National Laboratory.

Received: August 5, 2002
Revised: December 5, 2002

References

Abraham, R.T. (2001). Cell cycle checkpoint signaling through the ATM and ATR kinases. Genes Dev. *15*, 2177–2196.

Adams, P.D., Sellers, W.R., Sharma, S.K., Wu, A.D., Nalin, C.M., and Kaelin, W.G., Jr. (1996). Identification of a cyclin-cdk2 recognition motif present in substrates and p21-like cyclin-dependent kinase inhibitors. Mol. Cell. Biol. *16*, 6623–6633.

Bakkenist, C.J., and Kastan, M.B. (2003). DNA damage activates ATM through intermolecular autophosphorylation and dimer dissociation. Nature *421*, 499–506.

Banin, S., Moyal, L., Shieh, S., Taya, Y., Anderson, C.W., Chessa, L., Smorodinsky, N.I., Prives, C., Reiss, Y., Shiloh, Y., and Ziv, Y. (1998). Enhanced phosphorylation of p53 by ATM in response to DNA damage. Science *281*, 1674–1677.

Bell, D.W., Varley, J.M., Szydlo, T.E., Kang, D.H., Wahrer, D.C., Shannon, K.E., Lubratovich, M., Verselis, S.J., Isselbacher, K.J., Fraumeni, J.F., et al. (1999). Heterozygous germ line hCHK2 mutations in Li-Fraumeni syndrome. Science *286*, 2528–2531.

Blasina, A., Price, B.D., Turenne, G.A., and McGowan, C.H. (1999). Caffeine inhibits the checkpoint kinase ATM. Curr. Biol. *9*, 1135–1138.

Bravo, R., and Macdonald-Bravo, H. (1985). Changes in the nuclear distribution of cyclin (PCNA) but not its synthesis depend on DNA replication. EMBO J. *4*, 655–661.

Brown, E.J., and Baltimore, D. (2000). ATR disruption leads to chromosomal fragmentation and early embryonic lethality. Genes Dev. *14*, 397–402.

Bulger, M., Ito, T., Kamakaka, R.T., and Kadonaga, J.T. (1995). Assembly of regularly spaced nucleosome arrays by *Drosophila* chromatin assembly factor 1 and a 56 kDa histone-binding protein. Proc. Natl. Acad. Sci. USA *92*, 11726–11730.

Canman, C.E., Lim, D.S., Cimprich, K.A., Taya, Y., Tamai, K., Sakaguchi, K., Appella, E., Kastan, M.B., and Siliciano, J.D. (1998). Activation of the ATM kinase by ionizing radiation and phosphorylation of p53. Science *281*, 1677–1679.

Casper, A.M., Nghiem, P., Arlt, M.F., and Glover, T.W. (2002). ATR regulates fragile site stability. Cell *111*, 779–789.

Celis, J.E., and Celis, A. (1985). Cell cycle-dependent variations in the distribution of the nuclear protein cyclin proliferating cell nuclear antigen in cultured cells: subdivision of S phase. Proc. Natl. Acad. Sci. USA *82*, 3262–3266.

Cha, R.S., and Kleckner, N. (2002). ATR homolog Mec1 promotes fork progression, thus averting breaks in replication slow zones. Science *297*, 602–606.

Cliby, W.A., Roberts, C.J., Cimprich, K.A., Stringer, C.M., Lamb, J.R., Schreiber, S.L., and Friend, S.H. (1998). Overexpression of a kinase-inactive ATR protein causes sensitivity to DNA-damaging agents and defects in cell cycle checkpoints. EMBO J. *17*, 159–169.

Cortez, D., Wang, Y., Qin, J., and Elledge, S.J. (1999). Requirement of ATM-dependent phosphorylation of BRCA1 in the DNA damage response to double-strand breaks. Science *286*, 1162–1166.

Costanzo, V., Robertson, K., Bibikova, M., Kim, E., Grieco, D., Gottesman, M., Carroll, D., and Gautier, J. (2001). Mre11 protein complex prevents double-strand break accumulation during chromosomal DNA replication. Mol. Cell *8*, 137–147.

Couch, F.J., and Weber, B.L. (2000). Breast cancer. In The Metabolic and Molecular Bases of Inherited Disease, A. Beaudet, B. Childs, K. Kinzler, C. Scriver, S. Sly, D. Valle, and B. Vogelstein, eds. (New York: McGraw-Hill), pp. 999–1030.

de Klein, A., Muijtjens, M., van Os, R., Verhoeven, Y., Smit, B., Carr, A.M., Lehmann, A.R., and Hoeijmakers, J.H.J. (2000). Targeted disruption of the cell-cycle checkpoint gene ATR leads to early embryonic lethality in mice. Curr. Biol. *10*, 479–482.

Desany, B.A., Alcasabas, A.A., Bachant, J.B., and Elledge, S.J. (1998). Recovery from DNA replicational stress is the essential function of the S-phase checkpoint pathway. Genes Dev. *12*, 2956–2970.

Gatei, M., Scott, S.P., Filippovitch, I., Soronika, N., Lavin, M.F., Weber, B., and Khanna, K.K. (2000). Role for ATM in DNA damage-induced phosphorylation of BRCA1. Cancer Res. *60*, 3299–3304.

Gatei, M., Zhou, B.B., Hobson, K., Scott, S., Young, D., and Khanna, K.K. (2001). Ataxia telangiectasia mutated (ATM) kinase and ATM and Rad3 related kinase mediate phosphorylation of Brca1 at distinct and overlapping sites. In vivo assessment using phospho-specific antibodies. J. Biol. Chem. *276*, 17276–17280.

Hakem, R., de la Pompa, J.L., Sirard, C., Mo, R., Woo, M., Hakem, A., Wakeham, A., Potter, J., Reitmair, A., Billia, F., et al. (1996). The tumor suppressor gene Brca1 is required for embryonic cellular proliferation in the mouse. Cell *85*, 1009–1023.

Hall, C., Nelson, D.M., Ye, X., Baker, K., DeCaprio, J.A., Seeholzer, S., Lipinski, M., and Adams, P.D. (2001). HIRA, the human homologue of yeast Hir1p and Hir2p, is a novel cyclin-cdk2 substrate whose expression blocks S-phase progression. Mol. Cell. Biol. *21*, 1854–1865.

Han, M., Chang, M., Kim, U.J., and Grunstein, M. (1987). Histone H2B repression causes cell-cycle-specific arrest in yeast: effects on chromosomal segregation, replication, and transcription. Cell *48*, 589–597.

Kanda, T., Sullivan, K.F., and Wahl, G.M. (1998). Histone-GFP fusion protein enables sensitive analysis of chromosome dynamics in living mammalian cells. Curr. Biol. *8*, 377–385.

Kaufman, P.D., Kobayashi, R., Kessler, N., and Stillman, B. (1995). The p150 and p60 subunits of chromatin assembly factor I: a molecular link between newly synthesized histones and DNA replication. Cell *81*, 1105–1114.

Kaufman, P.D., Cohen, J.L., and Osley, M.A. (1998). Hir proteins are required for position-dependent gene silencing in *Saccharomyces cerevisiae* in the absence of chromatin assembly factor I. Mol. Cell. Biol. *18*, 4793–4806.

Khanna, K.K., and Jackson, S.P. (2001). DNA double-strand breaks: signaling, repair and the cancer connection. Nat. Genet. *27*, 247–254.

Kim, U.J., Han, M., Kayne, P., and Grunstein, M. (1988). Effects of histone H4 depletion on the cell cycle and transcription of *Saccharomyces cerevisiae*. EMBO J. *7*, 2211–2219.

Kodama, Y., Rothman, J.H., Sugimoto, A., and Yamamoto, M. (2002). The stem-loop binding protein CDL-1 is required for chromosome condensation, progression of cell death and morphogenesis in *Caenorhabditis elegans*. Development *129*, 187–196.

Kolodner, R.D., Putnam, C.D., and Myung, K. (2002). Maintenance of genome stability in *Saccharomyces cerevisiae*. Science *297*, 552–557.

Krawitz, D.C., Kama, T., and Kaufman, P.D. (2002). Chromatin assembly factor I mutants defective for PCNA binding require Asf1/Hir proteins for silencing. Mol. Cell. Biol. *22*, 614–625.

Lengauer, C., Kinzler, K.W., and Vogelstein, B. (1998). Genetic instabilities in human cancers. Nature *396*, 643–649.

Liu, C.Y., Flesken-Nikitin, A., Li, S., Zeng, Y., and Lee, W.H. (1996). Inactivation of the mouse Brca1 gene leads to failure in the morpho-

genesis of the egg cylinder in early postimplantation development. Genes Dev. *10*, 1835–1843.

Liu, Q., Guntuku, S., Cui, X.S., Matsuoka, S., Cortez, D., Tamai, K., Luo, G., Carattini-Rivera, S., DeMayo, F., Bradley, A., et al. (2000). Chk1 is an essential kinase that is regulated by Atr and required for the G(2)/M DNA damage checkpoint. Genes Dev. *14*, 1448–1459.

Loo, D.T., and Rillema, J.R. (1998). Measurement of cell death. Methods Cell Biol. *57*, 251–264.

Lopes, M., Cotta-Ramusino, C., Pellicioli, A., Liberi, G., Plevani, P., Muzi-Falconi, M., Newlon, C.S., and Foiani, M. (2001). The DNA replication checkpoint response stabilizes stalled replication forks. Nature *412*, 557–561.

Marheineke, K., and Krude, T. (1998). Nucleosome assembly activity and intracellular localization of human CAF-1 changes during the cell division cycle. J. Biol. Chem. *273*, 15279–15286.

Martini, E., Roche, D.M., Marheineke, K., Verreault, A., and Almouzni, G. (1998). Recruitment of phosphorylated chromatin assembly factor 1 to chromatin after UV irradiation of human cells. J. Cell Biol. *143*, 563–575.

Mertens, F., Johansson, B., Hoglund, M., and Mitelman, F. (1997). Chromosomal imbalance maps of malignant solid tumors: a cytogenetic survey of 3185 neoplasms. Cancer Res. *57*, 2765–2780.

Moggs, J.G., Grandi, P., Quivy, J.P., Jonsson, Z.O., Hubscher, U., Becker, P.B., and Almouzni, G. (2000). A CAF-1-PCNA-mediated chromatin assembly pathway triggered by sensing DNA damage. Mol. Cell. Biol. *20*, 1206–1218.

Nelson, D.M., Ye, X., Hall, C., Santos, H., Ma, T., Kao, G.D., Yen, T.J., Harper, J.W., and Adams, P.D. (2002). Coupling of DNA synthesis and histone synthesis in S-phase independent of cyclin/cdk2 activity. Mol. Cell. Biol. *22*, 7459–7472.

Oesterreich, S., Allredl, D.C., Mohsin, S.K., Zhang, Q., Wong, H., Lee, A.V., Osborne, C.K., and O'Connell, P. (2001). High rates of loss of heterozygosity on chromosome 19p13 in human breast cancer. Br. J. Cancer *84*, 493–498.

Osborn, A.J., Elledge, S.J., and Zou, L. (2002). Checking on the fork: the DNA-replication stress-response pathway. Trends Cell Biol. *12*, 509–516.

Quivy, J.-P., Grandi, P., and Almouzni, G. (2001). Dimerization of the largest subunit of chromatin assembly factor 1: importance in vitro and during *Xenopus* early development. EMBO J. *20*, 2015–2027.

Redon, C., Pilch, D., Rogakou, E., Sedelnikova, O., Newrock, K., and Bonner, W. (2002). Histone H2A variants H2AX and H2AZ. Curr. Opin. Genet. Dev. *12*, 162–169.

Sarkaria, J.N., Busby, E.C., Tibbetts, R.S., Roos, P., Taya, Y., Karnitz, L.M., and Abraham, R.T. (1999). Inhibition of ATM and ATR kinase activities by the radiosensitizing agent, caffeine. Cancer Res. *59*, 4375–4382.

Scully, R., Chen, J., Ochs, R.L., Keegan, K., Hoekstra, M., Feunteun, J., and Livingston, D.M. (1997). Dynamic changes of BRCA1 subnuclear location and phosphorylation state are initiated by DNA damage. Cell *90*, 425–435.

Sharp, J.A., Fouts, E.T., Krawitz, D.C., and Kaufman, P.D. (2001). Yeast histone deposition protein Asf1p requires Hir proteins and PCNA for heterochromatic silencing. Curr. Biol. *11*, 463–473.

Sheng, Z.M., Marchetti, A., Buttitta, F., Champeme, M.H., Campani, D., Bistocchi, M., Lidereau, R., and Callahan, R. (1996). Multiple regions of chromosome 6q affected by loss of heterozygosity in primary human breast carcinomas. Br. J. Cancer *73*, 144–147.

Shibahara, K., and Stillman, B. (1999). Replication-dependent marking of DNA by PCNA facilitates CAF-1-coupled inheritance of chromatin. Cell *96*, 575–585.

Smith, S., and Stillman, B. (1989). Purification and characterization of CAF-I, a human cell factor required for chromatin assembly during DNA replication in vitro. Cell *58*, 15–25.

Smith, S., and Stillman, B. (1991). Immunological characterization of chromatin assembly factor I, a human cell factor required for chromatin assembly during DNA replication in vitro. J. Biol. Chem. *266*, 12041–12047.

Sogo, J.M., Lopes, M., and Foiani, M. (2002). Fork reversal and ssDNA accumulation at stalled replication forks owing to checkpoint defects. Science *297*, 599–602.

Stillman, B. (1986). Chromatin assembly during SV40 DNA replication in vitro. Cell *45*, 555–565.

Sullivan, E., Santiago, C., Parker, E.D., Dominski, Z., Yang, X., Lanzotti, D.J., Ingledue, T.C., Marzluff, W.F., and Duronio, R.J. (2001). *Drosophila* stem-loop binding protein coordinates accumulation of mature histone mRNA with cell cycle progression. Genes Dev. *15*, 173–187.

Takai, H., Tominaga, K., Motoyama, N., Minamishima, Y.A., Nagahama, H., Tsukiyama, T., Ikeda, K., Nakayama, K., and Nakanishi, M. (2000). Aberrant cell cycle checkpoint function and early embryonic death in Chk1(−/−) mice. Genes Dev. *14*, 1439–1447.

Taniguchi, T., Garcia-Higuera, I., Xu, B., Andreassen, P.R., Gregory, R.C., Kim, S.T., Lane, W.S., Kastan, M.B., and D'Andrea, A.D. (2002). Convergence of the Fanconi anemia and ataxia telangiectasia signaling pathways. Cell *109*, 459–472.

Tercero, J.A., and Diffley, J.F. (2001). Regulation of DNA replication fork progression through damaged DNA by the Mec1/Rad53 checkpoint. Nature *412*, 553–557.

Tibbetts, R.S., Brumbaugh, K.M., Williams, J.M., Sarkaria, J.N., Cliby, W.A., Shieh, S.Y., Taya, Y., Prives, C., and Abraham, R.T. (1999). A role for ATR in the DNA damage-induced phosphorylation of p53. Genes Dev. *13*, 152–157.

Tibbetts, R.S., Cortez, D., Brumbaugh, K.M., Scully, R., Livingston, D., Elledge, S.J., and Abraham, R.T. (2000). Functional interactions between BRCA1 and the checkpoint kinase ATR during genotoxic stress. Genes Dev. *14*, 2989–3002.

Tyler, J.K. (2002). Chromatin assembly. Eur. J. Biochem. *269*, 2268–2274.

Tyler, J.K., Adams, C.R., Chen, S.R., Kobayashi, R., Kamakaka, R.T., and Kadonaga, J.T. (1999). The RCAF complex mediates chromatin assembly during DNA replication and repair. Nature *402*, 555–560.

Vogelstein, B., Lane, D., and Levine, A.J. (2000). Surfing the p53 network. Nature *408*, 307–310.

Vousden, K.H. (2002). Activation of the p53 tumor suppressor protein. Biochim. Biophys. Acta *1602*, 47–59.

Warbrick, E. (2000). The puzzle of PCNA's many partners. Bioessays *22*, 997–1006.

Weiss, R.S., Enoch, T., and Leder, P. (2000). Inactivation of mouse Hus1 results in genomic instability and impaired responses to genotoxic stress. Genes Dev. *14*, 1886–1898.

Xiao, Y., and Weaver, D.T. (1997). Conditional gene targeted deletion by Cre recombinase demonstrates the requirement for the double-strand break repair Mre11 protein in murine embryonic stem cells. Nucleic Acids Res. *25*, 2985–2991.

Zhu, J., Petersen, S., Tessarollo, L., and Nussenzweig, A. (2001). Targeted disruption of the Nijmegen breakage syndrome gene NBS1 leads to early embryonic lethality in mice. Curr. Biol. *11*, 105–109.

[Cell Cycle 2:3, 2-187, May/June 2003]; © 2003 Landes Bioscience

Extra Views

Coordination of S-Phase Events and Genome Stability

Xiaofen Ye
Peter D. Adams*

Fox Chase Cancer Center; W446; 7701 Burholme Avenue; Philadelphia, Pennsylvania USA

*Correspondence to: Peter D. Adams; Fox Chase Cancer Center; W446; 7701 Burholme Avenue; Philadelphia, Pennsylvania 19111 USA; Email: PD_Adams@fccc.edu

Received 04/14/03; Accepted 04/15/03

Previously published online as a Cell Cycle Paper in Press at:
http://www.landesbioscience.com/journals/cc/toc.php?volume=2&issue=3

KEY WORDS

Chromatin assembly, S-phase, Checkpoint, DNA damage, Histone synthesis

The lab of PDA is funded by grants from the NIH (GM62281), the DOD (DAMD17-02-1-0726) and a Scholar Award from the Leukemia and Lymphoma Society to PDA.

S-phase is defined as the time in the cell cycle when DNA synthesis occurs. However, it is also the time when histones are synthesized and when DNA, histones and non-histone chromatin proteins are assembled into mature chromatin. Chromatin is the physiological substrate for virtually all DNA-based transactions, such as transcription, repair, recombination and DNA synthesis itself. Moreover, a large amount of epigenetic information is stored in chromatin structure, as reflected by phenomena such as X chromosome inactivation and genetic imprinting.[1] In light of this, it seems likely that the S-phase events that build mature chromatin, namely DNA synthesis, histone synthesis and chromatin assembly, are very tightly controlled and coordinated with respect to each other.

Indeed, there is good evidence that S-phase events are tightly coordinated during S-phase. The pool of free histones in S-phase of somatic cells is thought to be very small and newly synthesized DNA is incorporated into nucleosomes within a few hundred base-pairs of the replication fork.[2,3] This indicates that the rates of DNA synthesis, histone synthesis and chromatin assembly are closely matched. Confirming this, it has long been known that inhibition of DNA synthesis with drugs such as hydroxyurea and aphidicolin causes a concerted inhibition of histone synthesis. In yeast this is due to repression of transcription but in mammalian cells it is largely due to rapid destabilization of histone mRNAs.[4]

However, until recently, there was little evidence to support the idea that DNA synthesis, histone synthesis and chromatin assembly are coupled in the reverse direction. In other words, that completion of DNA synthesis depends upon on-going histone synthesis and chromatin assembly. In fact, several lines of evidence from various model systems suggest that DNA synthesis in S-phase can occur in the absence of histone synthesis and chromatin assembly. First, in yeast none of the likely DNA synthesis-linked chromatin assembly factors identified to date, such as CAF-I, ASF1 or the Hir proteins, is essential for viability either alone or in combination.[5-7] Second, in vitro replication of plasmid DNA in mammalian cell extracts or *D. melanogaster* embryo extracts does not require chromatin assembly.[8,9] Third, yeast expressing histones from conditional promoters apparently replicate their entire genome when new histone synthesis and chromatin assembly are blocked.[10,11] Fourth, in *C. elegans* and *D. melanogaster* embryos the reduced histone synthesis caused by mutant alleles of SLBP has no obvious effect on DNA synthesis.[12] Fifth, although in *X. laevis* perturbation of CAF-I blocks development past the mid-blastula transition, it has no detectable effect on a somatic cell line.[13]

In contrast to these observations, Nelson et al. recently obtained evidence to indicate that in intact human cells on-going DNA synthesis is dependent upon continued histone synthesis and/or chromatin assembly.[14] Specifically, it was shown that repression of histone synthesis in human cells by ectopic expression of human HIRA, the human ortholog of two yeast repressors of histone transcription (Hir1p and Hir2p), inhibits DNA synthesis and causes arrest in S-phase.[14,15] One possible explanation of this result is that repression of histone synthesis inhibits chromatin assembly which, in turn, inhibits DNA synthesis. To test this idea Ye et al. inhibited the heterotrimeric **C**hromatin **A**ssembly **F**actor-I (CAF-I) complex that is responsible for assembling newly synthesized DNA into nucleosomes. To do this the authors made use of a fragment of the 150kDa subunit of CAF-I that seemed to be a good candidate for a dominant negative inhibitor of CAF-I, since it was known to bind to one essential subunit of the complex, p60CAF-I, but not to PCNA which targets CAF-I to replication foci.[16,17] Indeed, Ye et al. showed that, as expected, this fragment, p150C, inhibits CAF-I dependent chromatin assembly in vitro, disrupts the heterotrimeric CAF-I complex and displaces p60CAF-I from the sites of DNA synthesis in vivo.[18] However, p150C did not affect the characteristic punctate S-phase distribution of PCNA throughout the nucleus, indicating that it does not completely disrupt nuclear replication foci. Most significantly, ectopic expression of p150C blocked progression of cells into and through S-phase, in a manner that was virtually indistinguishable from

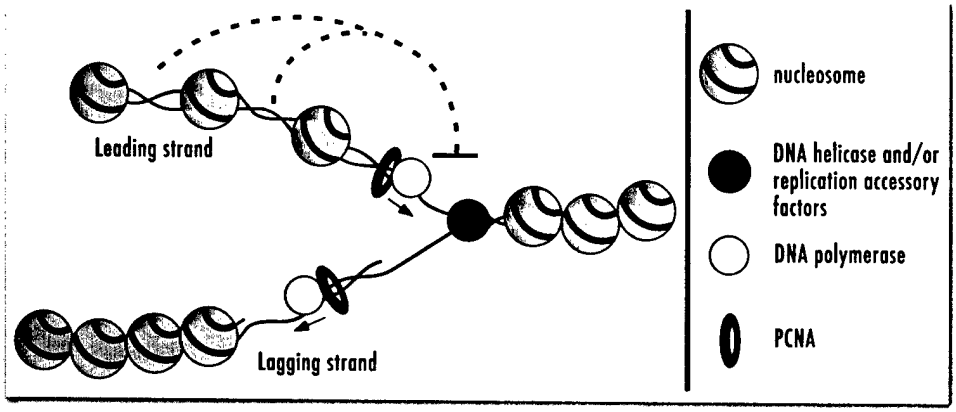


Figure 1. A putative chromatin assembly checkpoint. The figure shows a replication fork where nucleosome assembly has occurred normally on the lagging strand. In contrast, nucleosome assembly has been impaired on the leading strand, resulting in newly synthesized DNA with less than the normal complement of nucleosomes. This activates a checkpoint that prevents replication fork progression. Such a checkpoint can account for the experimental observation that chromatin assembly occurs within a few hundred base-pairs of fork passage and will facilitate the prompt and orderly assembly of chromatin behind the replication fork, thereby helping to preserve locus specific chromatin structures and epigenetic control of gene expression through S-phase.

ectopically expressed HIRA.[14,15,18] Taken together, the data suggest that inhibition of chromatin assembly, either due to repression of histone synthesis or direct inhibition of the chromatin assembly process itself, inhibits DNA synthesis in S-phase. Thus, three major processes in S-phase—DNA synthesis, histone synthesis and chromatin assembly—appear to be mutually dependent upon each other in intact mammalian cells.

What is the mechanism by which these three processes are linked in S-phase? In human cells inhibition of DNA synthesis triggers a rapid destabilization of histone mRNAs that is dependent upon a stem-loop structure within the 3'-UTR of the mRNA.[19,20] This stem-loop and 3'-UTR bind to a number of proteins involved in regulation of histone mRNA stability and processing, such as SLBP, the U7 snRNP and a zinc-finger protein, ZFP100 (see ref. 21 and refs. therein). However, the signal transduction pathway that presumably emanates from a stalled replication fork and ultimately triggers degradation of the mRNA is unknown. Interestingly, we found that hydroxyurea-induced destabilization of histone mRNAs is abolished by treatment of the cells with caffeine, an inhibitor of checkpoint activated ATR and ATM kinases.[22] This suggests that the well known genotoxic stress activated checkpoint pathways, centered on ATR and ATM, might trigger mRNA destabilization.[23]

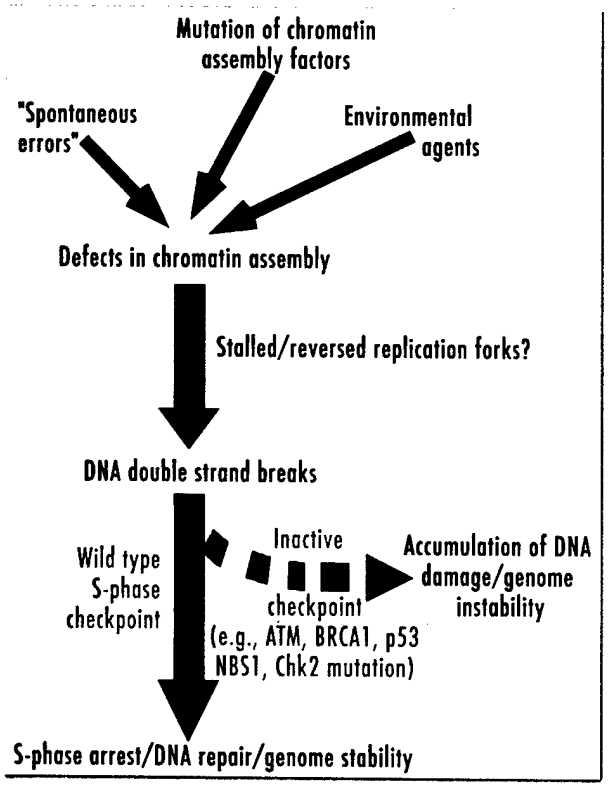


Figure 2. Defects in chromatin assembly cause DNA damage. Defects in chromatin assembly, arising spontaneously or due to mutation of chromatin assembly factors or the presence of environmental agents, cause DNA double strand breaks, likely due to inappropriate processing of stalled replication forks. In the presence of a wild type S-phase checkpoint, S-phase is arrested and DNA damage repaired, thus preserving genome integrity. In the absence of an S-phase checkpoint, due, for example, to any of several inactivating mutations found in human cancers, DNA damage persists and contributes to genome instability and, potentially, cancer.

One model to explain the converse coupling process, whereby inhibition of histone synthesis and/or chromatin assembly blocks DNA synthesis, invokes a putative "chromatin assembly checkpoint" (Fig. 1). According to this model, a failure to promptly incorporate newly synthesized DNA into chromatin results in activation of a checkpoint that prevents continued DNA synthesis. This checkpoint might be triggered by stretches of newly synthesized DNA with less then the normal complement of nucleosomes and would, presumably, facilitate the ordered assembly of chromatin structures behind the replication fork. Consistent with this idea, Kastan and coworkers showed recently that treatments which perturb chromatin structure without inducing DNA damage activate the ATM kinase.[24] This suggests that ATM and related kinases, such as ATR, are able to sense defects in chromatin structure. Consistent with p150C activating such a chromatin assembly checkpoint, the p150C-induced S-phase arrest was accompanied by checkpoint activation, as reflected by formation of nuclear ATR foci, stabilization and phosphorylation of p53 and dispersal of BRCA1 S-phase foci.[18] Moreover, efficient S-phase arrest induced by p150C was abolished in cells lacking ATR and ATM kinases, suggesting that the arrest requires activation of the S-phase checkpoint.

Although such a chromatin assembly checkpoint is an attractive idea which might help preserve chromatin-based epigenetic control of gene expression through S-phase, an alternative explanation for p150C-induced S-phase arrest was suggested by the observation that the p150C-induced S-phase arrest was accompanied by DNA double strand breaks. Such breaks are already well known to activate the S-phase checkpoint and cause cell cycle arrest and undoubtedly contribute to the arrest induced by p150C[23] (Fig. 2). But how do defects in chromatin assembly cause DNA double strand breaks? Most likely, the breaks result from inappropriate processing of stalled replication forks. Stalled replication forks can under-go fork reversal to form so-called "chicken-feet" that can, in turn, be resolved to form double strand breaks.[25,26] Defects in S-phase chromatin assembly

might cause stalling of replication forks due to obligate coupling of the chromatin assembly and DNA replication machineries, or because of steric problems caused by newly synthesized DNA that is not packaged into chromatin. Alternatively, inefficient chromatin assembly behind the replication fork might not increase stalling of replication forks but, instead, increase the frequency with which stalled forks form chicken-feet, the precursors of double strand breaks, or it might expose the newly synthesized DNA to nucleases.

Although this model perhaps sheds less light than the chromatin assembly checkpoint on the mechanism by which a cell coordinates DNA synthesis and chromatin assembly in a normal S-phase, it has significant implications for maintenance of genome stability. Kolodner and coworkers, in a series of elegant studies, have shown that the S-phase checkpoint is required in a normal S-phase, in the absence of exogenous DNA damaging agents, to prevent accumulation of genome instability.[27] In addition, numerous lines of evidence indicate that one role of the S-phase checkpoint is to stabilize replication forks, thus preventing their stalling and conversion to double strand breaks.[25,26,28] Thus, it seems likely that stalled replication forks are a potent source of genome instability in S-phase and one job of the S-phase checkpoint it to protect against this form of DNA damage. However, the causes of stalled replication forks are poorly understood. The results of Ye et al. suggest that defects in chromatin assembly, occurring spontaneously or as a result of genetic mutations or environmental toxins, are one source of stalled forks and double strand breaks.[18] One prediction of this model is that mutations that impair the function of chromatin assembly factors should cooperate with mutations that inactivate the S-phase checkpoint to promote genome instability and associated diseases, such as cancer. The S-phase checkpoint is known to be a frequent target of mutations in human cancer.[29-32] We are currently testing whether genes encoding chromatin assembly factors are also mutated in human cancers.

These two models, in which defects in chromatin assembly activate a chromatin assembly checkpoint or act as a source of DNA damage and genome instability, are not mutually exclusive. Indeed, one advantage of a chromatin assembly checkpoint might be that it would prevent a gross defect in chromatin structure from accumulating behind a replication fork, to a point where it causes fork stalling and a double strand break. Future experiments will determine whether a chromatin assembly checkpoint contributes to coordination of DNA synthesis and chromatin assembly in S-phase, and whether defects in S-phase chromatin assembly impact upon genome stability and human disease.

References.

1. Lee JT. Molecular Links between X-Inactivation and Autosomal Imprinting: X-Inactivation as a Driving Force for the Evolution of Imprinting? Curr Biol 2003; 13:R242-54.
2. DePamphilis ML, Wassarman, PM. Replication of eukaryotic chromosomes: a close-up of the replication fork. Annu Rev Biochem 1980; 49:627-66.
3. McKnight SL, Miller, OL, Jr. Electron microscopic analysis of chromatin replication in the cellular blastoderm Drosophila melanogaster embryo. Cell 1977; 12:795-804.
4. Osley MA. The regulation of histone synthesis in the cell cycle. Annu Rev Biochem 1991; 60:827-61.
5. Kaufman PD, Cohen JL, Osley MA. Hir proteins are required for position-dependent gene silencing in Saccharomyces cerevisiae in the absence of chromatin assembly factor I. Mol Cell Biol 1998; 18:4793-806.
6. Sharp JA, Fouts ET, Krawitz DC, Kaufman, PD. Yeast histone deposition protein Asf1p requires Hir proteins and PCNA for heterochromatic silencing. Curr Biol 2001; 11:463-73.
7. Tyler JK, Adams CR, Chen SR, Kobayashi R, Kamakaka RT, Kadonaga, JT. The RCAF complex mediates chromatin assembly during DNA replication and repair. Nature 1999; 402:555-60.
8. Bulger M, Ito T, Kamakaka RT, Kadonaga JT. Assembly of regularly spaced nucleosome arrays by Drosophila chromatin assembly factor 1 and a 56-kDa histone-binding protein. Proc Natl Acad Sci USA 1995; 92:11726-30.
9. Stillman B. Chromatin assembly during SV40 DNA replication in vitro. Cell 1986; 45:555-65.
10. Han M, Chang M, Kim UJ, Grunstein M. Histone H2B repression causes cell-cycle-specific arrest in yeast:effects on chromosomal segregation, replication, and transcription. Cell 1987; 48:589-97.
11. Kim UJ, Han M, Kayne P, Grunstein, M. Effects of histone H4 depletion on the cell cycle and transcription of Saccharomyces cerevisiae. Embo J 1988; 7:2211-9.
12. Sullivan E, Santiago C, Parker ED, Dominski Z, Yang X, Lanzotti DJ, Ingledue TC, Marzluff WF, Duronio RJ. Drosophila stem loop binding protein coordinates accumulation of mature histone mRNA with cell cycle progression. Genes Dev 2001; 15:173-87.
13. Quivy JP, Grandi P, Almouzni G. Dimerization of the largest subunit of chromatin assembly factor 1: importance in vitro and during Xenopus early development. Embo J 2001; 20:2015-27.
14. Nelson DM, Hall C, Santos H, Ma T, Kao GD, Yen TJ, Harper JW, Adams PD. Coupling of DNA synthesis and histone synthesis in S-phase independent of cyclin/cdk2 activity. Mol. Cell. Biol. 2002; 22:7459-72.
15. Hall C, Nelson DM, Ye X, Baker K, DeCaprio JA, Seeholzer S, Lipinski M, Adams PD. HIRA, the human homologue of yeast Hir1p and Hir2p, is a novel cyclin-cdk2 substrate whose expression blocks S-phase progression. Mol Cell Biol 2001; 21:1854-65.
16. Kaufman PD, Kobayashi R, Kessler N, Stillman B. The p150 and p60 subunits of chromatin assembly factor I:a molecular link between newly synthesized histones and DNA replication. Cell 1995; 81:1105-14.
17. Moggs JG, Grandi P, Quivy JP, Jonsson ZO, Hubscher U, Becker PB, Almouzni G. A CAF-1-PCNA-mediated chromatin assembly pathway triggered by sensing DNA damage. Mol Cell Biol 2000; 20:1206-18.
18. Ye X, Franco AA, Santos H, Nelson DM, Kaufman PD, Adams PD. Defective S-phase chromatin assembly causes DNA damage, activation of the S-phase checkpoint and S-phase arrest. Molecular Cell 2003; 11:341-351.
19. Pandey NB, Marzluff WF. The stem-loop structure at the 3' end of histone mRNA is necessary and sufficient for regulation of histone mRNA stability. Mol Cell Biol 1987; 7:4557-9.
20. Levine BJ, Chodchoy N, Marzluff WF, Skoultchi AI. Coupling of replication type histone mRNA levels to DNA synthesis requires the stem-loop sequence at the 3' end of the mRNA. Proc Natl Acad Sci USA 1987; 84:6189-93.
21. Dominski Z, Erkmann JA, Yang X, Sanchez R, Marzluff WF. A novel zinc finger protein is associated with U7 snRNP and interacts with the stem-loop binding protein in the histone pre-mRNP to stimulate 3'-end processing. Genes Dev 2002; 16:58-71.
22. Hall C, Adams PD. Unpublished data. 2003.
23. Abraham RT. Cell cycle checkpoint signaling through the ATM and ATR kinases. Genes Dev 2001; 15:2177-96.
24. Bakkenist CJ, Kastan, MB. DNA damage activates ATM through intermolecular autophosphorylation and dimer dissociation. Nature 2003; 421:499-506.
25. Osborn AJ, Elledge SJ, Zou L. Checking on the fork:the DNA-replication stress-response pathway. Trends Cell Biol 2002; 12:509-516.
26. Sogo JM, Lopes M, Foiani M. Fork reversal and ssDNA accumulation at stalled replication forks owing to checkpoint defects. Science 2002; 297:599-602.
27. Kolodner RD, Putnam CD, Myung K. Maintenance of genome stability in Saccharomyces cerevisiae. Science 2002; 297:552-7.
28. Cha RS, Kleckner N. ATR homolog Mec1 promotes fork progression, thus averting breaks in replication slow zones. Science 2002; 297:602-6.
29. Khanna KK, Jackson SP. DNA double-strand breaks: signaling, repair and the cancer connection. Nat Genet 2001; 27:247-54.
30. Bell DW, Varley JM, Szydlo TE, Kang DH, Wahrer DC, Shannon KE, Lubratovich M, Verselis SJ, Isselbacher KJ, Fraumeni JF, Birch JM, Li FP, Garber JE, Haber DA. Heterozygous germ line hCHK2 mutations in Li-Fraumeni syndrome. Science 1999; 286:2528-31.
31. Vogelstein B, Lane D, Levine AJ. Surfing the p53 network. Nature 2000; 408:307-10.
32. Taniguchi T, Garcia-Higuera I, Xu B, Andreassen PR, Gregory RC, Kim ST, Lane WS, Kastan MB, D'Andrea AD. Convergence of the fanconi anemia and ataxia telangiectasia signaling pathways. Cell 2002; 109:459-72.

Molecular Cell, Vol. 11, 283–291, February, 2003, Copyright ©2003 by Cell Press

Previews

Histone Deposition at the Replication Fork: A Matter of Urgency

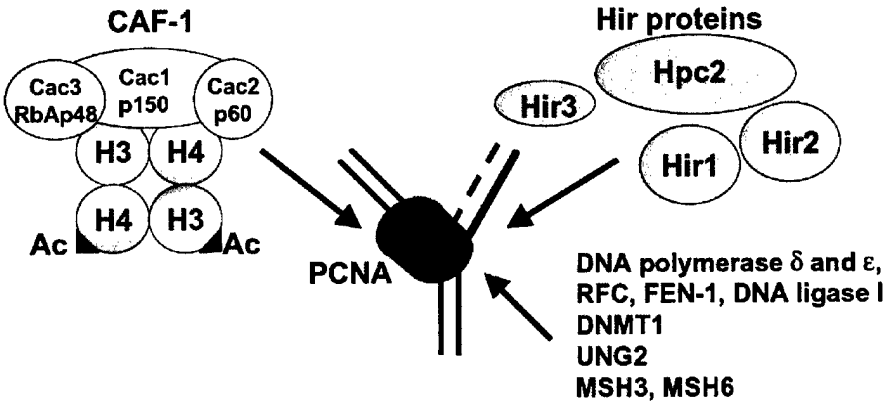
In this issue of *Molecular Cell*, Ye et al. provide a biological rationale for rapid histone deposition behind the replication fork. They show that defects in nucleosome assembly lead to DNA double-strand breaks and S phase arrest. Their results have important implications for the maintenance of genome integrity in proliferating cells.

During DNA replication, half of the nucleosomes are formed by transfer of preexisting histones onto newly synthesized DNA, a reaction known as parental histone segregation. In contrast, the other half of the nucleosomes are formed from newly synthesized and acetylated histones through a pathway known as de novo nucleosome assembly. Classical experiments using psoralen crosslinking and electron microscopy to visualize chromatin structure revealed that nucleosomes reform within ~250 bp behind the replication fork (Sogo et al., 1986). Thus, both histone segregation and de novo histone deposition occur almost as soon as enough DNA is available to form nucleosomes.

De novo nucleosome assembly occurs through a stepwise mechanism whereby histones H3 and H4, which contact the central portion of the DNA, are deposited first. Histones H2A and H2B, which contact the DNA near the ends of the nucleosome core, are added subsequently. Histones H3/H4 form stable complexes with a number of assembly factors that escort them to the replication fork and facilitate their regulated deposition onto DNA. Chromatin Assembly Factor 1 (CAF-1) is a three-subunit protein (p150/p60/RbAp48) that brings histones to the DNA replication fork via a direct interaction with Proliferating Cell Nuclear Antigen (PCNA), a DNA polymerase processivity factor that forms a sliding clamp around DNA (see Figure). In *S. cerevisiae*, four Hir proteins function in a pathway that is genetically redundant with CAF-1, but their precise role in nucleosome assembly is not clear. Unlike CAF-1, Hir proteins also act as repressors of histone genes. The respective contributions of CAF-1 and Hir proteins to nucleosome assembly can be blocked by separate mutations in PCNA (Sharp et al., 2001; Zhang et al., 2001). In addition to its role in DNA replication and nucleosome assembly, PCNA also serves as a platform to attract a variety of other enzymes that are constitutively present at the replication fork. Given that histones often impede access to DNA, the fact that nucleosome assembly, replication, and repair proteins all utilize interactions with PCNA suggests an important biological role for rapid histone deposition behind the replication fork.

In this issue of *Molecular Cell*, Ye et al. (2003) provide compelling evidence that CAF-1-dependent histone deposition behind the fork is necessary to prevent spontaneous DNA damage in human cells. The authors use dominant-negative mutants of the p150 subunit that are incapable of binding to PCNA. Overexpression of these p150 mutants inactivates CAF-1 by sequestering the other subunits of the protein away from sites of DNA replication. The resulting defect in de novo nucleosome assembly leads to DNA double-strand breaks and S phase arrest. The arrest is a typical DNA damage response involving phosphorylation of histone H2A-X and p53 by the ATM and ATR kinases. Overriding the arrest with caffeine (an inhibitor of ATM/ATR) leads to cell death. It seems unlikely that inactivation of CAF-1 could trigger DNA damage and S phase arrest via a mechanism independent of its role in nucleosome assembly. Using a different approach to inhibit nucleosome assembly, the authors previously showed that repression of histone synthesis by overexpression of HIRA (a human homolog of yeast Hir1 and Hir2) also causes DNA damage checkpoint activation and S phase arrest (Nelson et al., 2002; Ye et al., 2003). Yeast cells undergoing S phase in the absence of histone synthesis also lose viability, but they do not exhibit a prominent delay in DNA replication (Kim et al., 1988). However, the possibility that these cells may accumulate DNA double-strand breaks has not been explored.

Interestingly, expression of dominant-negative p150 only results in a modest increase in chromatin sensitivity to micrococcal nuclease. This suggests that the chromatin alterations due to the absence of CAF-1 may be restricted to short regions behind replication forks. Alternatively, as is the case in *S. cerevisiae*, an alternative pathway for histone deposition at the replication fork may compensate for the absence of CAF-1 in human cells. In any case, the results suggest that even subtle defects in global chromatin structure, perhaps confined to the vicinity of replication forks, are sufficient to cause DNA damage and S phase arrest. It is not immediately obvious how the lack of CAF-1-dependent nucleosome assembly, which occurs behind the replication fork, leads to formation of double-strand breaks. Perhaps the action of nucleases may normally be restricted by rapid histone deposition behind the replication fork. Alternatively, there may be crosstalk between fork progression and events that needs to occur behind the replication fork. Defects in nucleosome assembly behind the fork may elicit a response that delays or stalls the replication fork. Although pausing the replication fork to enable the nucleosome assembly machinery to "catch up" may be generally beneficial, excessive pausing may occasionally lead to DNA damage. A third possibility may be that, in the absence of CAF-1-dependent nucleosome assembly, stochastic fork stalling does not occur more frequently than in wild-type cells, but stalled forks are more often processed into double-strand breaks when the DNA behind the fork is free. In *S. cerevisiae*, Mec1 (a homolog of human ATM/ATR) and a downstream kinase known as Rad53 (CHK2 in human cells) function to prevent the processing of stalled replication forks into reversed forks and DNA double-strand breaks (Cha and Kleckner, 2002; Sogo et al., 2002). The results of Ye et al. (2003) suggest that the local chromatin environment



Two Parallel Pathways for Nucleosome Assembly Converge on PCNA at the Replication Fork

The names of the CAF-1 subunits in *S. cerevisiae* and human cells are shown. Yeast Hir1 and Hir2 are related to human HIRA. Many replication proteins (DNA polymerases, FEN-1, RF-C, ligase I) and enzymes involved in maintenance of DNA methylation (DNMT1), uracil excision repair (UNG2), and mismatch repair (MSH3 and MSH6) also contain PCNA binding motifs similar to that of CAF-1.

may also influence the conversion of stalled replication forks into damaged DNA.

The discovery that histone deposition must occur rapidly behind the fork has important ramifications. It is known that histone overexpression has deleterious effects on mitotic chromosome segregation (Meeks-Wagner and Hartwell, 1986) and that inhibition of DNA replication leads to disappearance of histone mRNAs. This implies that cells maintain a very delicate balance between histone and DNA synthesis. This is not a trivial task given that replication origin usage and S phase duration can vary dramatically among cell types. For example, some embryonic cells and activated B cells can replicate their DNA at a furious pace. In addition, most cell types are likely to experience a drastic decline in total rates of DNA synthesis as they progress from early S phase (when many replication forks are active) to late S phase (when fewer replication forks are active). The problem is compounded by the fact that many forms of DNA damage are known to slow down rates of elongation and prevent initiation of new replication forks (Tercero and Diffley, 2001). How cells balance histone and DNA synthesis in response to such abrupt, but accidental, changes in rates of DNA replication is not known. Future research will no doubt reveal a wealth of regulatory mechanisms that enable cells to maintain an optimal balance between histone and DNA synthesis during both normal S phase progression and in response to DNA damage.

Alain Verreault
Cancer Research UK
London Research Institute
Clare Hall Laboratories
Blanche Lane
South Mimms
Herts EN6 3LD
United Kingdom

Selected Reading

Cha, R.S., and Kleckner, N. (2002). Science *297*, 602–606.

Kim, U.J., Han, M., Kayne, P., and Grunstein, M. (1988). EMBO J. *7*, 2211–2219.

Meeks-Wagner, D., and Hartwell, L.H. (1986). Cell *44*, 43–52.

Nelson, D.M., Ye, X., Hall, C., Santos, H., Ma, T., Kao, G.D., Yen, T.J., Harper, J.W., and Adams, P.D. (2002). Mol. Cell. Biol. *22*, 7459–7472.

Sharp, J.A., Fouts, E.T., Krawitz, D.C., and Kaufman, P.D. (2001). Curr. Biol. *11*, 463–473.

Sogo, J.M., Lopes, M., and Foiani, M. (2002). Science *297*, 599–602.

Sogo, J.M., Stahl, H., Koller, T., and Knippers, R. (1986). J. Mol. Biol. *189*, 189–204.

Tercero, J.A., and Diffley, J.F.X. (2001). Nature *412*, 553–557.

Ye, X., Franco, A.A., Santos, H., Nelson, D.M., Kaufman, P.D., and Adams, P.D. (2003). Mol. Cell *11*, this issue, 341–351.

Zhang, Z., Shibahara, K., and Stillman, B. (2001). Nature *408*, 221–225.

Plant Defense: One Post, Multiple Guards?!

***Arabidopsis* RIN4 is a key bacterial virulence target that is guarded by the resistance (R) protein RPM1. Two recent studies suggest that another R protein, RPS2, also guards RIN4. Bacterial avirulence (Avr) effectors AvrB, AvrRpm1, and AvrRpt2 alter this key protein. R proteins RPM1 and RPS2 recognize the altered status and initiate a defense-signaling response. The guard hypothesis is in!**

The recognition of pathogen-derived avirulence (Avr) effectors by plant resistance (R) proteins triggers a defense response in the host that often results in rapid

HIGHLIGHTS

HIGHLIGHTS ADVISORS

UELI AEBI
UNIVERSITY OF BASEL, SWITZERLAND

TOM L. BLUNDELL
UNIVERSITY OF CAMBRIDGE, UK

JOAN S. BRUGGE
HARVARD MEDICAL SCHOOL, BOSTON, MA, USA

PASCALE COSSART
INSTITUT PASTEUR, PARIS, FRANCE

PAMELA GANNON
CELL AND MOLECULAR BIOLOGY ONLINE

SUSAN M. GASSER
UNIVERSITY OF GENEVA, SWITZERLAND

JEAN GRUENBERG
UNIVERSITY OF GENEVA, SWITZERLAND

ULRICH HARTL
MAX-PLANCK-INSTITUTE, MARTINSRIED, GERMANY

STEPHEN P. JACKSON
WELLCOME/CRC INSTITUTE, CAMBRIDGE, UK

WALTER NEUPERT
MUNICH UNIVERSITY, GERMANY

TONY PAWSON
SAMUEL LUNENFELD RESEARCH INSTITUTE, TORONTO, CANADA

NORBERT PERRIMON
HARVARD MEDICAL SCHOOL, BOSTON, MA, USA

THOMAS D. POLLARD
YALE UNIVERSITY, NEW HAVEN, CT, USA

JOHN C. REED
THE BURNHAM INSTITUTE, LA JOLLA, CA, USA

ANNE RIDLEY
LUDWIG INSTITUTE FOR CANCER RESEARCH, LONDON, UK

KAREN VOUSDEN
BEATSON INSTITUTE FOR CANCER RESEARCH, GLASGOW, UK

CHROMATIN

A fork load

In a recent study, Peter Adams and colleagues showed that repression of histone synthesis causes S-phase arrest. Adams' group now reports, in *Molecular Cell*, that the assembly of nucleosomes onto newly synthesized DNA near the replication fork — rather than histone synthesis *per se* — is tightly coupled to DNA synthesis in human cells.

Adams and co-workers showed that a dominant-negative mutant of the p150CAF-I subunit of the heterotrimeric chromatin-assembly factor CAF-I inhibits CAF-I-dependent chromatin assembly in an *in vitro* assay. The mutant protein (called HA-p150C) disrupts the endogenous CAF-I complex *in vivo* by preventing the binding of wild-type p150CAF-I to the p60CAF-I subunit. As a result, p60CAF-I can no longer associate with chromatin at replication foci.

To investigate what happens at the site of DNA synthesis, the replication fork, Adams and co-workers transfected cells with a plasmid encoding HA-p150C, and found that DNA synthesis and progression through S phase were blocked. Co-expression of the wild-type p150CAF-I subunit abolished cell-cycle arrest, confirming that perturbation of CAF-I inhibits DNA synthesis. As expected, the cells that were arrested in S phase had an abnormal chromatin structure that was hypersensitive to micrococcal nuclease digestion (that is, the DNA is more accessible to the nuclease, because the chromatin structure is perturbed).

S-phase arrest can be caused by DNA damage. So was this the case for defective chromatin assembly-induced arrest? As it turned out, yes — Adams and colleagues found that HA-p150C-expressing cells contained fragmented DNA, and that histone H2AX was phosphorylated, presumably, in response to double-strand breaks.

What causes the inhibition of DNA synthesis and S-phase arrest? The S-phase checkpoint protects the cell's genome integrity during S phase, and is activated in response to DNA damage. So, Adams and colleagues reckoned that inhibition of chromatin assembly might activate the S-phase checkpoint, which is indeed what happens.

H2AX is a substrate for ATR and/or ATM, both S-phase checkpoint kinases, and the authors showed that ATR nuclear foci, the sites of active ATR, are present preferentially in HA-p150C-expressing cells arrested in S phase. Other substrates of ATR and ATM — p53 and BRCA1 — were also activated. Phosphorylation of p53 was induced, and dispersed BRCA1 foci, which are typical of activated BRCA1, were enriched in HA-p150C-expressing cells that contained ATR foci.

The authors propose that "...defects in S-phase chromatin assembly cause double-strand breaks due to stalling and inappropriate processing of replication forks". This indicates that defects in chromatin assembly, combined with inactivation of the S-phase checkpoint, might promote genome instability, and Adams and colleagues are now testing whether genes encoding chromatin-assembly factors might be mutated in human cancers.

Arianne Heinrichs

References and links

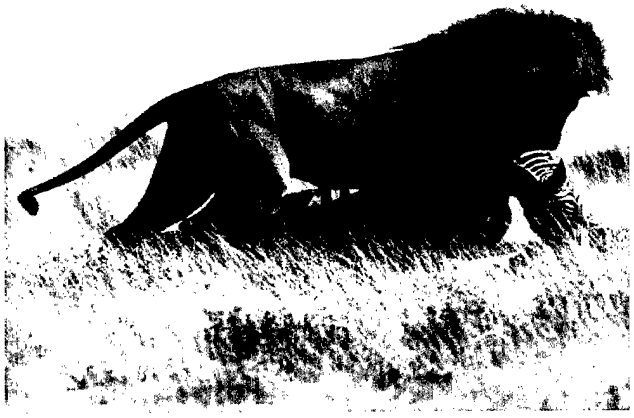
ORIGINAL RESEARCH PAPER Ye, X. *et al.* Defective S phase chromatin assembly causes DNA damage, activation of the S phase checkpoint, and S phase arrest. *Mol. Cell* **11**, 341–351 (2003)

FURTHER READING Nelson, D. M. *et al.* Coupling of DNA synthesis and histone synthesis in S-phase independent of cyclin/cdk2 activity. *Mol. Cell. Biol.* **22**, 7459–7472 (2002)

© 2003 Nature Publishing Group

VIRAL TRANSMISSION

Deadly contact



Transmission of the human T-cell lymphotropic virus type I (HTLV-I) seems to require cell–cell contact — the contribution to infection by cell-free HTLV-I virions is minimal. In their report in *Science*, Igakura *et al.* now show that HTLV-I is transmitted across cell–cell junctions after polarizing the cytoskeleton of the infected cell at sites of cell–cell contact.

The authors first looked at the distribution of the viral (Gag) core proteins and the glycoprotein envelope (Env) protein in isolated infected T cells, and in uninfected cells that had conjugated with HTLV-I-infected cells. After 40 minutes, they saw a strong polarization of both proteins from around the cell periphery in infected cells to the area of cell–cell contact in conjugates — a significant finding because the nucleocapsid p15 Gag protein is known to incorporate the retroviral genome into virions. In addition, another Gag protein, p19, was detected in the 'uninfected' cells of the conjugates, which might represent the initial establishment of HTLV-I infection.

Following on from the detection of p15 at the cell–cell contacts, Igakura *et al.* studied the localization of the HTLV-I genome. The HTLV-I nucleic acid was not polarized in single infected T cells, but it accumulated at cell–cell junctions of infected–uninfected conjugated cell pairs, similar to what was seen for the Gag and Env proteins. As was also seen for the Gag p19 protein, viral nucleic acid was later transferred to the 'uninfected' cell.

What is the cause of this asymmetrical localization? The authors noticed that polarized Gag proteins at the cell–cell junctions were frequently closely juxtaposed to a reorientated microtubule-organizing centre (MTOC). As nocodazole, which depolymerizes microtubules, inhibited the cell–cell accumulation and subsequent cell transfer of Gag, this implicates microtubule dynamics in the polarization of Gag. In addition, Igakura *et al.* showed that MTOC

PLANT DEVELOPMENT

Channelling elongation

Everybody knows that to grow, plants need minerals and water from the soil, which they obtain through roots and root hairs. The formation of these structures requires cell expansion — by way of elongation — which, in turn, needs calcium (Ca^{2+}) acquisition. But, until now, what regulated the Ca^{2+} influx wasn't so obvious. Research led by Liam Dolan's group, though, has pinpointed the production of reactive oxygen species (ROS) by an NAPDH oxidase in the activation of Ca^{2+} channels in elongating root cells.

Because *Arabidopsis thaliana rhd2* mutants develop very short root hairs and stunted roots, and are defective in Ca^{2+} uptake, the authors decided to clone the gene encoding RHD2. They found that the gene — *At5g51060* — had previously been defined as *Arabidopsis thaliana* respiratory burst oxidase homologue C (*AtrbohC*). Rather unsurprisingly, as implied by the name, the *At*rbohC protein and other *At*rbohs are homologous to the $gp91^{phox}$ subunit of the mammalian NAPDH oxidase that catalyses ROS production.

What, then, is the connection between RHD2/*At*rbohC and growth? ROS production was reduced by ~50% in root apices from *rhd2* mutants compared with wild-type apices. Normally, ROS are present as the root hair emerges as a bulge and further increase as the elongation rate goes up. Adding an inhibitor of NADPH oxidase to the apices of wild-type plants prevented ROS accumulation, the elongation of root-hair bulges and the extension rate of the primary root, thereby phenocopying the *rhd2* mutant.

The authors then tried the opposite approach. Could ROS applied to *rhd2*-mutant root-hair bulges induce root-hair growth? Indeed it could. Application of the most reactive ROS, hydroxyl radicals ($OH^{\bullet}$), to *rhd2*-mutant root-hair bulges restored root-hair growth, although the growth lacked the polarity found in wild-type hairs. Moreover, this was coincident with a rapid increase in the cytoplasmic levels of Ca^{2+} ($[Ca^{2+}]_c$), which was blocked in the presence of 0.1 mM Gd^{3+}, a Ca^{2+}-channel antagonist.

These data implicated ROS in the increase of $[Ca^{2+}]_c$ by Ca^{2+} influx, so the next step was to see if plasma-membrane Ca^{2+} channels could be activated by ROS. Within a few minutes of $OH^{\bullet}$ treatment, a Ca^{2+}-permeable, inwardly rectifying, hyperpolarization-activated conductance was detected in protoplasts from the elongation zone epidermis. This was again blocked by 0.1 mM Gd^{3+}, which also decreased the root elongation rate, as did a Ca^{2+} chelator. Because *rhd2* mutants and wild-type cells didn't differ significantly in their current amplitudes, the *rhd2* mutation seems not to affect the ROS-mediated channel sensitivity or the number of channel proteins. In root-hair apical spheroplasts, $OH^{\bullet}$ activated a Ca^{2+}-, Ba^{2+}- and TEA^{+}-permeable, inwardly rectifying, hyperpolarization-activated conductance.

So in protoplasts from the elongation zone epidermis and apical spheroplasts, ROS is involved in cell elongation by activating Ca^{2+} channels. The influx of Ca^{2+} is likely to modulate actin dynamics and other growth processes, and this mechanism could well extend to all plant cells. As the mammalian $gp91^{phox}$ is regulated by Rac, the authors propose that RHD2/*At*rbohC could be similarly controlled by Rac-like proteins in plants — ROPs.

Katrin Bussell

References and links

ORIGINAL RESEARCH PAPER Foreman, J. *et al.* Reactive oxygen species produced by NADPH oxidase regulate plant cell growth. *Nature* 2003 March 27 (DOI: 10.1038/nature01485)

WEB SITE

Liam Dolan's laboratory: http://www.jic.bbsrc.ac.uk/science/cdb/dolanwebpage.htm

© 2003 Nature Publishing Group